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Author(s)	Ikema, Yasunari; Tohyama, Harukazu; Yamamoto, Ei; Kanaya, Fuminori; Yasuda, Kazunori
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***Ex vivo* infiltration of fibroblasts into the tendon deteriorates the mechanical properties of tendon fascicles but not those of tendon bundles**

Yasunari Ikema (a, c); Harukazu Tohyama (a); Ei Yamamoto (b); Fuminori Kanaya (c); and Kazunori Yasuda (a)

a) Department of Sports Medicine and Joint Reconstruction Surgery, b) Department of Mechanical Engineering, School of Biology-Oriented Science and Technology, Kinki University, Wakayama 649-6493, Japan, and c) Department of Orthopedic Surgery, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa, 903-0215, Japan

Address Correspondence to:

Department of Sports Medicine and Joint Reconstruction Surgery,

Hokkaido University School of Medicine,

Kita-15 Nishi-7, Sapporo 060-8638, Japan

Tel: 81 (Japan)-11-706-7211,

Fax: 81(Japan)-11-706-7822

[Abstract]

Background: After ligament reconstruction, mechanical deterioration of the grafted tendon is observed with revascularization and cellular proliferation. However, the effect of cellular proliferation on the mechanical properties of the tendon matrix has not been fully understood.

Methods: Cultured fibroblasts derived from the rabbit patellar tendon were seeded around an acellular rabbit patellar tendon that had undergone freeze-thaw treatment. At time-zero, 3, and 6 weeks after seeding the cells, we evaluated cellular distribution in the tendon using a confocal laser microscope and the mechanical evaluations of the tendon fascicles and the tendon bundles.

Findings: The confocal laser microscopic analysis showed fibroblast infiltration *ex vivo* into the acellular tendon matrix. We could not find significant effects of the cellular infiltration on the tangent modulus of the tendon bundle, although the *ex vivo* cellular infiltration significantly reduced the modulus of the tendon fascicle. In addition, the tangent modulus of the incubated tendon without fibroblasts significantly decreased with time, particularly in the tendon bundle levels.

Interpretation: The findings of this study suggested that the effects of *ex vivo* cellular infiltration on the mechanical properties of the tendon bundles are relatively small,

compared with its striking effect on the tendon fascicles.

Key Words: fibroblast, cellular infiltration, ligament reconstruction, patellar tendon

1. Introduction

The fate of transplanted tendons for ligament reconstruction has been extensively studied (Clancy et al., 1981; Arnoczky et al., 1982; Amiel et al., 1986). These studies have shown that the graft to reconstruct the ligament undergoes dramatic changes in its structure. These changes can be divided into four stages: (i) avascular necrosis, (ii) revascularization, (iii) cellular proliferation, and (iv) remodeling (Amiel et al., 1986). In the overall process, mechanical deterioration of the grafted tendon is observed (Ballock et al., 1989). However, the mechanism of the mechanical deterioration of the grafted tendon has not been fully understood. We have reported that extrinsic cell infiltration and revascularization from the surrounding tissues accelerate the deterioration in the mechanical properties of the patellar tendon matrix after intrinsic fibroblast necrosis *in vivo* using the *in situ* frozen-thawed rabbit patellar tendon model (Tohyama and Yasuda, 2000). However, we could not isolate the effects of cellular infiltration into the tendon. The *ex vivo* approach may be one option to identify the effect of cellular infiltration into the tendon on the mechanical properties of the tendon. No *ex vivo* studies have been conducted to clarify the effect of extrinsic cell infiltration on the mechanical properties of the tendon matrix.

Natural tendon tissues have a hierarchical structure composed of bundles,

fascicles, fibrils, and fibers (Kastelic et al., 1978). Yamamoto and his coworkers (1999) developed a testing system for the evaluation of mechanical properties of collagen fascicles of the patellar tendon and found that the differences in tensile properties between fascicles and tendon bundles are attributable to mechanical interaction among the fascicles. Therefore, there is a possibility that extrinsic cell infiltration affects not only the material properties of collagen fascicles but also the mechanical interaction among the fascicles.

In the present study, we hypothesized that the *ex vivo* infiltration of tendon fibroblasts into the tendon significantly deteriorates not only the material properties of collagen fascicles of the tendon but also the mechanical interaction among the fascicles. The purpose of this study is to test this hypothesis by the *ex vivo* experiment. In the present study, we evaluated the matrix properties at two different levels in the collagen hierarchical structure: a fascicle level having a diameter of 300 μm and a bundle level having a width of 2.5 mm.

2. Materials and Methods

2.1. Animals for experiments

Thirty-six skeletally mature female Japanese White rabbits with an average

weight of 3.0 kg (SD: 0.1 kg) were used. All surgical procedures on the animals were carried out in the Institute of Animal Experimentation, Hokkaido University School of Medicine, under the Rules and Regulations of the Animal Care and Use Committee, Hokkaido University School of Medicine.

2.2. Experimental design

Table 1 shows the experimental design of the present study. In each animal, the patellar tendon was harvested from the bilateral knees. The right tendon was used to harvest the tendon fibroblasts and the left tendon was used to create an acellular tendon. Each left tendon underwent freeze-thaw treatment to necrotize cells and was then divided into two portions along the fiber direction for experiment and control specimens. For experiment specimens, cultured fibroblasts derived from each right patellar tendon were seeded around one acellular specimen that had been preserved after the freeze-thaw treatment. As a control without the fibroblast seeding, we incubated the other acellular specimens in the same manner. Twelve specimens were evaluated immediately after the seeding. The remaining 24 specimens were incubated after seeding, and 12 specimens each were evaluated at 3 and 6 weeks after seeding. At each period, six out of the 12 specimens were longitudinally divided into two portions along the fiber direction of the

tendon. One portion of each tendon was used for evaluation of cellular distribution using a confocal laser microscope and the other portion was used for mechanical evaluation of the tendon fascicles. The remaining six specimens at each period were used for mechanical evaluation of the tendon bundles.

2.3. Preparation of fibroblasts

The patellar tendons were harvested under aseptic conditions and placed individually in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The outer synovial layer was removed by sharp dissection and these tendons were cut into 2- to 5-mm pieces that were then placed in dishes (Naginani et al., 1992). The dishes were incubated at 37°C in a humidified, 5% CO₂ atmosphere (95% air) in a cell-culture incubator. A confluent monolayer formed in 2 weeks. Cells from the first passage, >95% fibroblast-like as confirmed by microscopic analysis, were used for all experiments.

2.4. Preparation of acellular tendon specimens

The left patellar tendons harvested from the animals were immersed in liquid nitrogen for 1 minute and then thawed in saline solution at 37°C to kill the intrinsic

fibroblasts in the patellar tendon. This procedure was repeated five times (King et al., 1995). Each tendon was then stored at -80°C until the experiment. Before the experiment, each tendon was partially thawed overnight at 4°C and thawed to completion at room temperature. Each tendon was divided into two portions along the fiber direction for experiment and control specimens. Each portion was then trimmed into 2-to-3 mm (thickness) x 15 mm (length) block, while we did not change its thickness, which was approximately 2-3 mm.

2.5. Ex vivo infiltration of fibroblasts into the acellular tendon

Subconfluent fibroblasts from the first passage were detached from the dishes by treatment for 2 minutes with trypsin-EDTA. Cultured fibroblasts were seeded into collagen gel (CELLGEN, KOKEN Co., Tokyo, Japan) (Kino-Oka et al., 2005) at a final concentration of 5×10^6 cells/ml (Figure 1). The acellular tendons were incubated in collagen gel with cultured fibroblasts in a 60-mm diameter culture dish and completely gelled by incubation at 37°C for 10 minutes before being overlaid with DMEM containing 10% FBS. Then, each tendon in collagen gel with cultured fibroblasts was incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 or 6 weeks. As a control, each acellular tendon in collagen gel without fibroblasts was simultaneously incubated in

the same conditions.

2.6. Evaluation of cellular distribution in the tendon

Immediately after the specimens were brought out from culture dishes, the specimens were frozen and stored at -80°C until the following examinations. The frozen specimens were partially thawed overnight at 4°C and thawed to completion at room temperature. Each tendon was divided into two portions for the evaluation of cellular distribution in the tendon and the mechanical evaluation of collagen fascicles. For cryosectioning, the tendons were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose in phosphate-buffered saline (PBS), and embedded in OCT (optimum cutting temperature) compound before cryosectioning at 25- μ m thickness in the sagittal direction. These slices were stained in 1 ml PBS containing 0.5 μ g propidium iodide (Molecular Probes, Eugene, OR, USA) for 30 minutes. We evaluated the cellular distribution in the tendon with a confocal laser microscope (MRC-1024; BIO-MED Laboratories, Tokyo, Japan). In addition, we counted the total number of infiltrated cells in one unit volume using software installed in the confocal laser microscope. In this analysis, we counted the cells in the central 2-mm portion of the tendon throughout its entire depth.

2.7. Mechanical testing for the tendon fascicles

Collagen fascicles with a diameter of approximately 300 μm and a length of 15 mm were very carefully dissected with a surgical knife in parallel to the axis of the patellar tendon. The cross-sectional area of the collagen fascicle was measured with the following non-contact method, which was developed by Yamamoto et al. (1999). Briefly, the lateral image of a collagen fascicle immersed in physiological saline solution at 37°C was enlarged by a low-magnification microscope (SMZ-2Z, Nikon, Tokyo), taken with a CCD camera (WV-BD400, Panasonic, Tokyo), and then processed with a video dimension analyzer (Percept Scope C3160, Hamamatsu Photonics, Tokyo, Japan). The diameter was measured from 36 directions, while the fascicle was intermittently rotated with a stepping motor at the angular interval of 5°. The cross-sectional area was calculated from averaging these diameters, assuming that the cross-section is circular. These measurements were done at a 2-mm position proximal from the middle, and at a 2-mm position distal to the middle. An average cross-sectional area was obtained from these two sets of measurements.

After the cross-sectional area measurement, we evaluated the tangent modulus of the collagen fascicles in the patellar tendons using a micro-tensile tester that was designed by Yamamoto et al. (1999) (Figure 2). Acrylic blocks were attached to the both

ends with cyanoacrylate adhesive. One of the blocks was attached to a load cell (LVS-1KA, Kyowa, Tokyo, Japan) and the other one was attached to the crosshead of the linear stage (LU09200AKL1-P5Z0, Nihonseiko, Tokyo, Japan). Tensile tests were carried out by moving the stage with a microprocessor-controlled stepping motor at the speed of 10 mm/min (UPD566TG30-A, Oriental Motor, Tokyo, Japan). For the measurement of strain, two markers were drawn on a collagen fascicle with a stain (nigrosine) about 5 mm apart; the strain measured with this method is the strain on the specimen surface (Yamamoto et al., 1999). The distance between the markers was measured with the above-mentioned video dimension analyzer using its tracking function. According to our pilot study, the total error including electrical noise is +/- 0.36%. For the gauge length of specimens in the present study, 5 mm, the total error is estimated to be 0.18 mm. During tensile testing, specimens were immersed in physiological saline solution at 37°C. Tensile load and the distance between the above-mentioned two markers were recorded on a personal computer (PC386-GE, Epson, Nagano, Japan). The tangent modulus of the tendon fascicles was defined as the slope of the stress-strain curve between 2% and 5% strain using a least-square method.

2.8. Mechanical testing for the tendon bundles

Each patellar tendon matrix was cut along the longitudinal axis with a specially designed cutter that had stainless-steel razor blades at 2.5-mm intervals. The cross-sectional area of the tendon was measured by the same non-contact method as the mechanical testing for collagen fascicles. After cross-sectional measurement, the ends of the tendon were put between two acrylic plates on which sandpaper was pasted. Then, the tendon was cast between the two plates using cyanoacrylic acid resin. Each specimen was mounted on a set of specially designed grips attached to a tensile tester (PTM-250 W; Orientec, Tokyo, Japan). The specimen and grips were immersed in 37°C saline solution during testing. After a preload of 0.5 N had been applied for 10 min, the specimen was subjected to 10 cycles of loading and unloading between elongation limits of 0 and 0.5 mm at a rate of 20 mm/min. The deformation of 0.5 mm was approximately 0.5% strain. Following this preconditioning, the specimen was stretched until failure at the same rate. Strain in the tendon substance was determined with the video dimension analyzer, using the two stained gauge-length markers. The tensile load and the displacement measured by the video dimension analyzer were recorded with an X–Y recorder. The tangent modulus of the tendon bundles was defined as the slope of the stress-strain curve between 2% and 4% strain using a least-square method.

2.9. Statistical Analysis

We statistically compared the total number of cells in a unit volume of the tendon matrix, the tensile strength and tangent modulus of the collagen fascicles, and the tangent modulus of the tendon bundles between the experiment and the control specimens at each period using unpaired *t*-tests. The one-way analysis of variance (ANOVA) was performed to assess the effect of incubation time. The significance limit was set at $P=0.05$.

3. Results

3.1. Cellular distribution in the patellar tendon matrix

Confocal laser microscopic images showed that fibroblasts infiltrated into the tendon *ex vivo* over time (Figure 3). On the other hand, no fibroblasts were found in the control tendon that had been incubated in collagen gel without cells throughout the experiment period. At 3 and 6 weeks, the total numbers of cells per unit volume of the experimental specimen were significantly higher than those of the control specimen (3 weeks: $P=0.001$; 6 weeks: $P=0.001$), while there were no significant differences in the total numbers of cells per unit volume between the experiment and the control tendon matrix specimens at time-0 ($P=0.549$)(Figure 4). The ANOVA showed the significant effect of the incubation time on the total number of cells per unit volume of the experiment

specimens but not on that of the control specimens (the experiment specimens: $P = 0.001$; the control specimens: $P = 0.485$).

3.2. Mechanical properties of the tendon

During tensile testing for collagen fascicles, all specimens failed at a portion between the two marks placed on the specimen. The averaged stress-strain relation curves are shown in Figure 5. At 3 and 6 weeks, the tangent modulus of the experiment specimen was significantly lower than that of the control specimen (3 weeks: $P = 0.003$, 6 weeks: $P = 0.048$), while there were no significant differences between the experiment and the control tendon matrix specimens at time-zero ($P = 0.543$)(Figure 6-a). In the experimental group with seeding cells, the average values of the tangent modulus at 3 weeks and 6 weeks were 54% and 36% of the average value at time-zero. The tangent modulus of the control specimen without cells at 3 and 6 weeks was significantly lower than that at time-zero (3 weeks: $P = 0.002$, 6 weeks: $P = 0.001$). There were no significant differences between 3 weeks and 6 weeks. In the control group without seeding cells, the average values of the tangent modulus at 3 weeks and 6 weeks were 93% and 56% of the average value at time-zero. The tangent modulus of the control specimen without cells at 6 weeks was significantly lower than those at time-zero and 3 weeks (time-zero: $P = 0.003$, 3

weeks: $P = 0.010$). There were no significant differences between time-zero and 3 weeks.

Concerning mechanical evaluation of the tendon bundles, most specimens failed at the grip in tensile testing (Table 1). There were no significant differences in the tangent modulus between the experiment and the control specimens (Figure 6-b). In the experimental group with seeding cells, the average values of the tangent modulus at 3 weeks and 6 weeks were 32% and 27% of that at time-zero. The tangent modulus of the experimental specimen with cells at 3 and 6 weeks was significantly lower than that time-zero (3 weeks: $P = 0.001$, 6 weeks: $P = 0.001$), while there were no significant differences between 3 weeks and 6 weeks. In the control group without seeding cells, the average values of the tangent modulus at 3 weeks and 6 weeks were 30% and 19% of that at time-zero. The tangent modulus of the control specimen without cells at 3 and 6 weeks was significantly lower than that at time-zero (3 weeks: $P = 0.001$, 6 weeks: $P = 0.001$), while there were no significant differences between 3 weeks and 6 weeks.

4. Discussion

The confocal laser microscopic analysis in this study indicated that the authors' culture technique successfully induced fibroblast infiltration *ex vivo* into the tendon matrix. This study demonstrated that the *ex vivo* cellular infiltration significantly reduced the tangent

modulus of the tendon fascicles, although we could not find significant effects of the cellular infiltration on the tangent modulus of the tendon bundles. In addition, we showed that the tangent modulus of the incubated tendons significantly decreased with time, even in the patellar tendon without fibroblast infiltration, particularly in the tendon bundle levels.

It is important to discuss how cellular infiltration deteriorated the mechanical properties of the tendon fascicles. Recently, Ritty and Herzog (2003) reported that tendon fibroblasts increased the production of matrix metalloproteinases (MMPs)- 2 and 9 in response to the attachment to type-I collagen matrix. MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) are thought to be the primary gelatinases (Bramono et al., 2004). As part of the cell-mediated remodeling of a tissue rich in fibrillar collagen, gelatinases would be expected to participate in matrix catalysis. Therefore, gelatinases might be up-regulated during *ex vivo* infiltration of tendon fibroblasts into the tendon matrix, which was mainly composed of type-I collagen, and might weaken collagen fascicles of the tendon.

In the present study, we found that the *ex vivo* effects of cellular infiltration on the tendon bundles were smaller than its effects on the tendon fascicles. The mechanical properties of the tendon bundles are considered to depend not only on the properties of the tendon fascicles but also on the mechanical interactions among the fascicles. In the

present *ex vivo* experiment, we did not apply any tension to the patellar tendon specimen. Yamamoto et al. (2000) reported that stress shielding significantly deteriorated the transverse mechanical properties of the patellar tendon, suggesting that mechanical deprivation reduced mechanical interactions among the collagen fascicles of the patellar tendon. Therefore, the effects of cellular infiltration on the mechanical deterioration of the tendon bundle may be masked by the drastic effects of stress deprivation on the patellar tendon. On the other hand, under 3-week stress-deprivation conditions, the average tangent modulus of the collagen fascicles with cellular infiltration was 54% of that of normal collagen fascicles, while the average tangent modulus of the collagen fascicles without cellular infiltration was 93% of that of normal collagen fascicles. These findings indicate that the effect of cellular infiltration on tendon fascicles is considered to be greater than the effect of 3-week stress deprivation. We believe that the effect of cellular infiltration on the material properties of the tendon fascicles is significant even under stress-deprived conditions, although the stress deprivation may mask the effect of cellular infiltration at the level of the tendon bundle as we stated above.

There are some limitations in the present study. The first limitation is related to the mechanical evaluation of the tendon bundles. As Table 2 shows, we found avulsion failures at the grip during tensile testing for the tendon bundles. Therefore, we should not

ignore problems associated with gripping the specimens incorporating tendon bundles. In the present study, we excluded the data of tensile strength and strain at failure from further analysis because there is a possibility that we might underestimate the tensile strength and the strain at failure of the tendon bundles. However, the tangent modulus of the normal patellar tendons in the present study, approximately 750 MPa, is similar to the tangent modulus of the normal patellar tendons in our previous study that measured mechanical properties of the patellar tendons by the tensile tests of the bone-tendon-bone complexes (Tohyama et al., 2003). We speculate that our strain measurement at the tendon substance using the video dimension analyzer minimized errors for the determination of the tangent modulus of the tendon bundles by problems associated with gripping the specimens. The second limitation is related to the evaluation of the cellular distribution in the tendon matrix. Before the evaluation of the cellular distribution in the tendon matrix, we devitalized all cells in the tendon specimens and dyed cells with propidium iodide for confocal laser microscopy. The reason why we did not attempt to dye living cells in the specimens is that our freeze-thaw treatment during sample preparation for confocal laser microscopy might affect the viability of the cells in the tendon specimens. We confirm that cells were not found in the tendon specimen without seeding cultured cells (the control group). Therefore, all cells in the experimental tendon

specimen are considered to originate from the seeded cells.

Concerning clinical relevance, this study suggested that we should take the striking deterioration in the mechanical properties of the tendon bundles by stress deprivation and mechanical deterioration of the tendon fascicles in response to *ex vivo* cellular infiltration into account when we intend to develop tissue-engineered tendon tissues in the near future (Dunn et al., 1995; Vunjak-Novakovic et al., 2004). Therefore, we should find a solution to prevent mechanical deterioration of tissue-engineered tendon tissues during cellular infiltration *ex vivo*.

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Table 1: Experimental design

Experimental step	Experimental group	Control group
1. Preparation of fibroblasts	● Explant culture for 36 right PTs	(-)
2. Preparation of acellular tendon matrix specimens	● Multiple freeze/thaw procedure for 36 left PTs	
3. <i>Ex vivo</i> infiltration of fibroblasts into tendon matrix	● Incubation of 36 acellular PTs with cultured fibroblasts from same animals	● Incubation of 36 acellular PTs without cells
4. Evaluation of cellular distribution in the tendon (time-0, 3 weeks, and 6 weeks, n=6 for each)	● Confocal laser microscope analysis	
5. Mechanical testing for the tendon fascicles (time-0, 3 weeks, and 6 weeks, n=6 for each)	● Preparation of tendon fascicles: a diameter 300 μ m and a length 15 mm ● Cross-sectional area measurement ● Tensile tests using a micro-tensile tester	
6. Mechanical testing for the tendon bundles (time-0, 3 weeks, and 6 weeks, n=6 for each)	● Preparation of tendon bundles: 2.5 mm (width) x 15 mm (length) x 2-to-3 mm (thickness) ● Cross-sectional area measurement ● Tensile tests using a conventional tensile tester	

PT: patellar tendon

Table 2: Failure modes of tensile tests for the tendon bundle specimens

Group	Experiment			Control		
	Time-0	3 weeks	6 weeks	Time-0	3 weeks	6 weeks
Avulsion at the grip	6	5	5	6	5	3
Substance failure	0	1	1	0	1	3

Figure Legends

Figure 1: Experimental procedure for *ex vivo* infiltration of fibroblasts into tendon matrix.

Cultured fibroblasts were seeded into collagen gel. The acellular tendon matrix was incubated in collagen gel with cultured fibroblasts in a culture dish and completely gelled before being overlaid with culture medium.

Figure 2: A micro-tensile tester used to determine the mechanical properties of the collagen fascicles.

Figure 3: Confocal laser microscopic images of fibroblast distribution in the acellular tendon matrix after incubation in collagen gel with (experiment) and without cultured fibroblasts (control) *ex vivo*.

Figure 4: The total number of cells in a unit volume of the tendon matrix after *ex vivo* incubation in collagen gel with cultured fibroblasts (experiment) and in collagen gel alone (control).

Figure 5: Averaged stress-strain curves of collagen fascicles of the tendon after incubation in collagen gel with cultured fibroblasts (experiment) (a) and without cultured fibroblasts (control) (b).

Figure 6: The tangent modulus values of collagen fascicles (a) and tendon bundles (b) of the tendon after incubation in collagen gel with (experiment) and without cultured

fibroblasts (control).

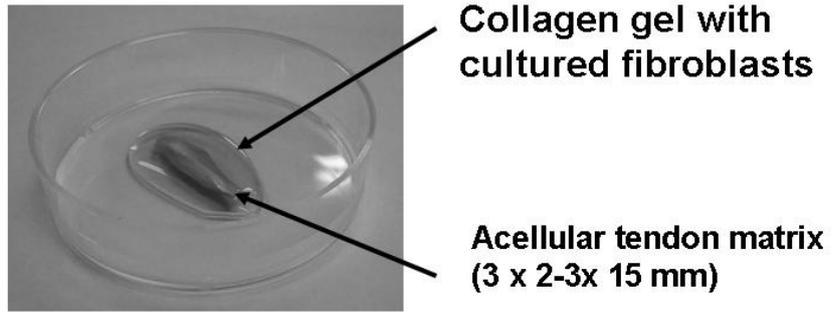


Figure 1

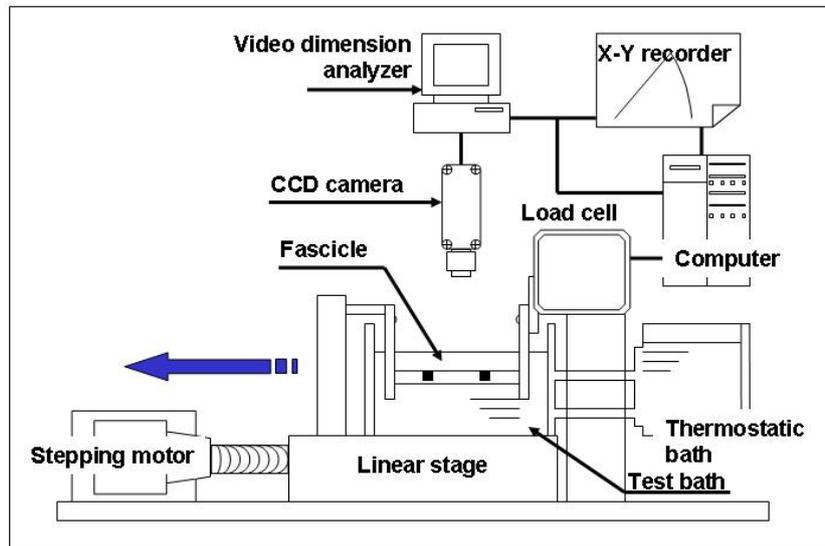


Figure 2

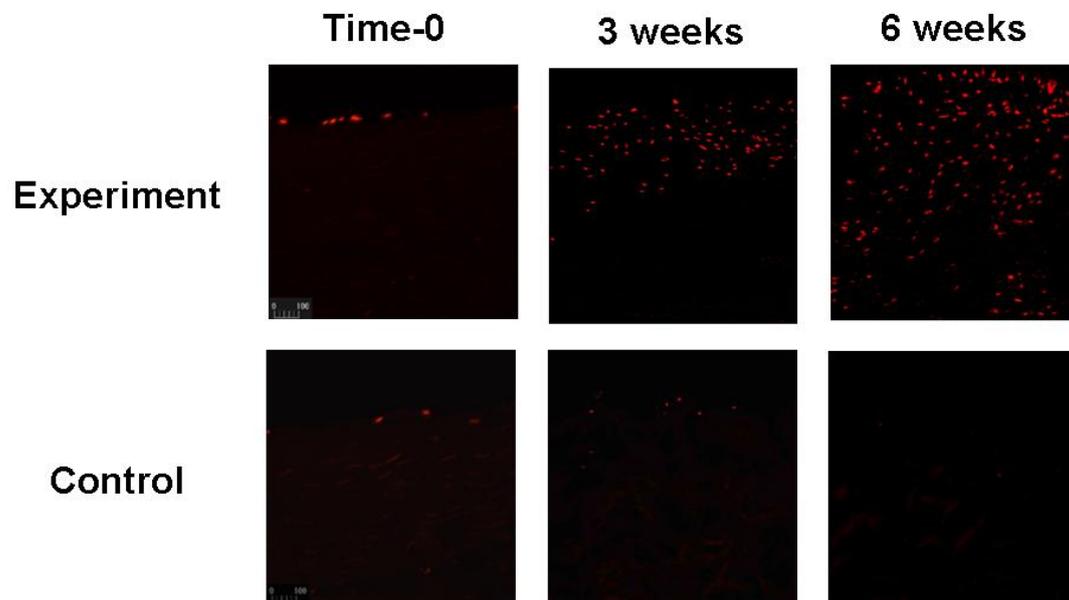


Figure 3

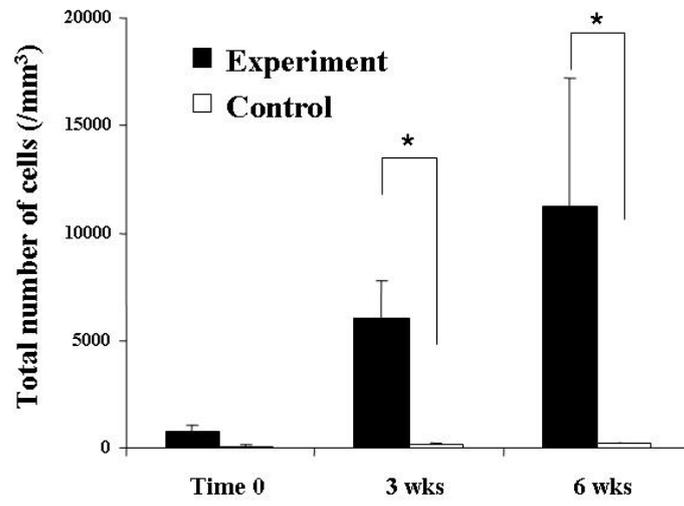
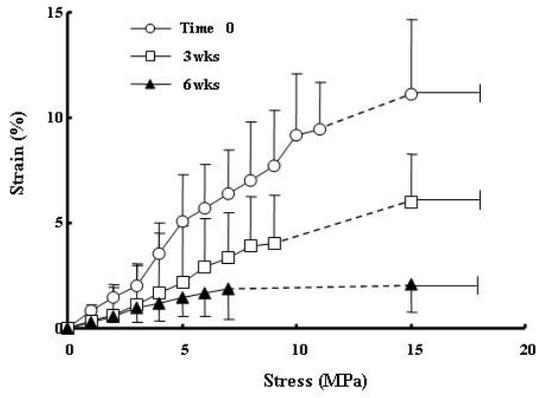


Figure 4

a.



b.

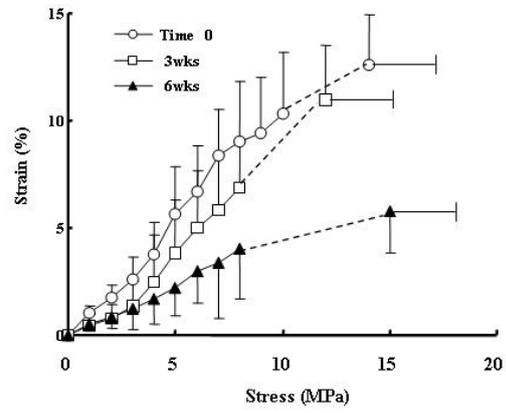


Figure 5

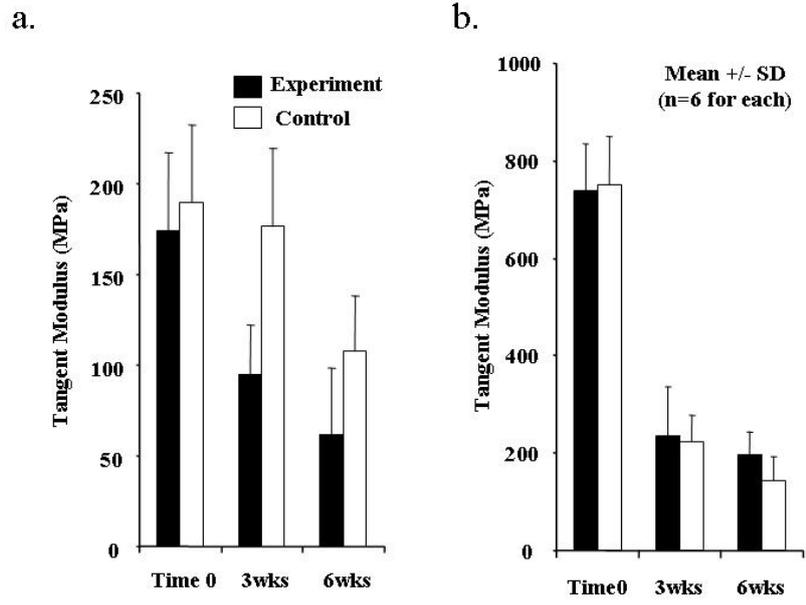


Figure 6