

Mechanical Training Biochamber For Engineered Ligoid Constructs

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Abstract:

In the United States alone, an estimated 14 million tendons and ligaments are damaged annually incurring a total medical cost of well over \$5 billion. Current treatment options for the repair of ligament and/or tendon damage are less than ideal, ranging from cadaver allografts to synthetic replacements, with patellar autografts as the standard of care. However, each treatment is associated with complications, including donor site morbidity, disease transmission, systemic incompatibility, and/or material failure. As a result, engineered autologous replacement tissues are desirable, from a clinically relevant cell source such as bone marrow stromal cells (BMSC). While previous reports have shown promise in developing self-organized tendon constructs in static culture, these constructs displayed the mechanical and histological characteristics of immature tissue¹. We hypothesized that such constructs, when subjected *in vitro* to a mechanical training regime similar to that experienced by native adult anterior cruciate ligament (ACL), would present superior mechanical and histological properties to those maintained in static culture only. In addition to mechanical training, we hypothesized that perfusion would facilitate the assembly of larger and more mature tissues. Toward these ends, we constructed a custom training biochamber capable of being perfused, in which we subjected self-organized constructs to a compound loading regime of 90° rotation coupled with 10% strain at a rate of 0.5 Hz. Cultured constructs generally presented a well-organized collagenous extracellular matrix occupying more than 70% of the cross-sectional area of representative constructs, composed principally of type I collagen, in a roughly tubular structure. Following mechanical training (n=9), collagen content was significantly higher than control constructs (n=16). Trained and perfused constructs (n=6) presented an increase in tissue diameter, and constructs receiving perfusion but not training (n=20) demonstrated an increased force to failure. Ligoids that were trained and perfused (n=6) demonstrated a lower stress to failure than controls due to the increased diameter without a commensurate increase in force. In conclusion, perfusion increased ultimate force and training decreased ultimate stress. More collagen was measured in trained ligoids but the training regime was too intense for that increase to translate into improved mechanics in these immature tissues.

Methods and Results:

Cell Isolation and Culture Conditions:

1. Digested porcine femur marrow in 300U/mL collagenase type I at 37°C for 40 min followed by 3 washes in basal Ham's F12 medium.
2. Expanded adherent BMSC in Growth Medium (GM: 79% αMEM with nucleosides, 20% FBS, 1% pen/strep, supplemented with 518μM L-ascorbic acid 2-phosphate, 430μM L-proline, and 670μM glycine).
3. Dish preparation: pinned 2 heavily frayed silk suture anchors (size 0, 6mm length) 12mm apart in the center of 60mm culture dishes coated with polydimethylsiloxane (PDMS), with frayed ends facing each other.
4. Laminin coating: air-dried a 5μg/cm² laminin layer (dissolved in DPBS) onto the PDMS surface, then rinsed salt crystals away with 2 DPBS washes. Added Preincubation Medium (PM: Ham's F12 with 20% FBS and 1% pen/strep), UV sterilized, and stored in 37°C/5%CO₂ incubator for 3-5 days.

Ligoid Construct Culture:

1. Aspirated off PM, and seeded 0.3 x 10⁶ cells per dish in GM.
2. Cultured in GM in 37°C/5%CO₂ incubator refreshing medium 3 times per week.
3. When the resulting monolayer began to delaminate from the dish perimeter, medium was changed to Differentiation Medium ("DM"; 93% αMEM without nucleosides, 5% horse serum, 1% L-glutamine, 1% pen/strep, supplemented with proline, glycine, and L-ascorbic acid 2 phosphate as above). All subsequent culture was conducted with freshly supplemented DM.
4. Five days after ligoid formation, constructs were divided into 4 treatment groups as described below.

Treatment Groups:

1. **Controls:** Grown in static culture after being repinned into a larger dish (5 ligoids per dish), with manual batch media feeds of 20mL every 24 hours.
2. **Trained:** Mounted in custom training biochambers and adjusted to 11-12mm gage length. Biochambers were programmed to deliver compound loading of 90° rotation coupled with 10% strain, cycling at 0.5Hz (Fig. 1), for 10 minutes every 24 hours or continuously over that period. Medium refreshed daily with 3mL DM.
3. **Perfused:** Mounted in a custom perfusion chamber with 5 ligoids per chamber, and subjected to a flow rate of 40mL/min which reversed direction every 12 minutes. Fed manually with 20mL every 24 hours.
4. **Trained and perfused:** Trained as described previously, with biochambers connected to a perfusion system programmed to deliver a constant flow rate of 40mL/min, reversing direction every 12 minutes, with a manual media feed of 10mL every 24 hours.

Characterization and analysis were conducted 14 days after construct formation.

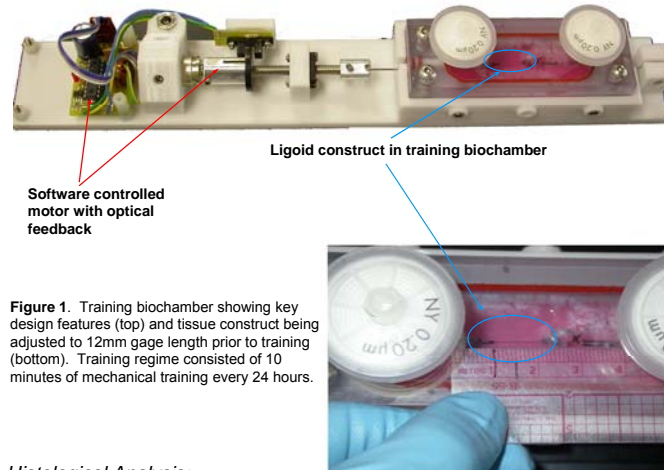


Figure 1. Training biochamber showing key design features (top) and tissue construct being adjusted to 12mm gage length prior to training (bottom). Training regime consisted of 10 minutes of mechanical training every 24 hours.

Histological Analysis:

Ligoids were fixed overnight in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, sectioned (6μm), and stained using Masson's Trichrome. Semi-quantitative percent collagen content was assessed using AnalySIS image analysis software.

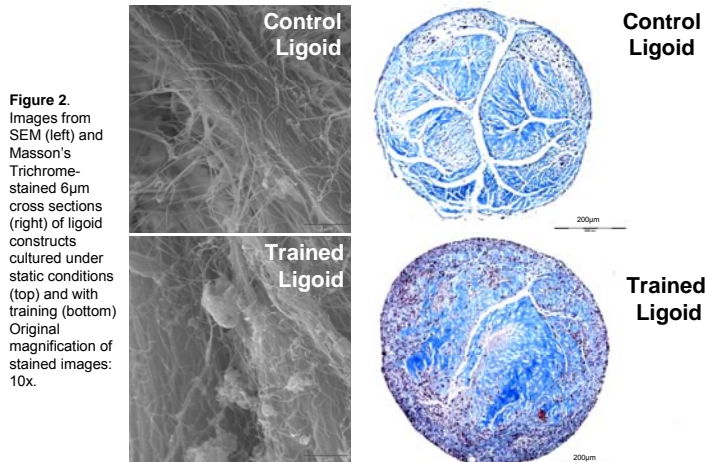
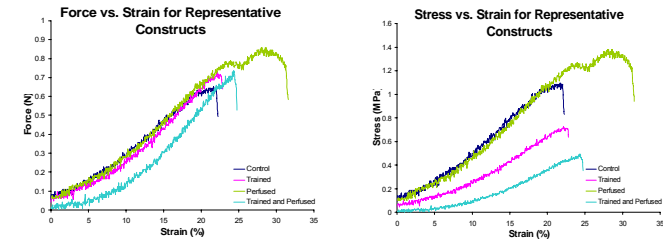


Figure 2. Images from SEM (left) and Masson's Trichrome-stained 6μm cross sections (right) of ligoid constructs cultured under static conditions (top) and with training (bottom). Original magnification of stained images: 10x.

Mechanical Testing:

Representative ligoids were mechanically tested to failure in tension at a strain rate of ≤1% per second using an EnduraTec ELF3200 with a 50lb. load cell.



Statistical Analysis:

Mechanical and collagen content data from ligoids were compared using a one-way ANOVA followed by Dunn's method of comparing ranked sums, using SigmaStat3.1 software. Differences (relative to control) were considered significant when $p < 0.05$.

Table 1. Characterization of engineered ligament constructs. Parameters significantly different from control values are shaded in blue and designated with ***. Data are presented as mean ± standard error.

Mechanical Property	Controls (n=12)	Trained (n=6)	Perfused (n=13)	Trained and perfused (n=6)
Diameter (mm)	0.79 ± 0.07	1.20 ± 0.26	0.94 ± 0.04	1.44 ± 0.09 *
Ultimate Tensile Force (N)	0.532 ± 0.040	0.561 ± 0.144	0.978 ± 0.116 *	0.659 ± 0.044
Ultimate Tensile Stress (MPa)	1.47 ± 0.35	0.60 ± 0.14	1.47 ± 0.20	0.43 ± 0.06 *
Ultimate Strain (%)	24.8 ± 1.4	25.0 ± 5.2	29.9 ± 2.6	26.6 ± 4.4
Tangent Modulus (MPa)	8.45 ± 1.78	3.39 ± 0.72	8.08 ± 1.29	3.16 ± 0.69
Histological Property	Controls (n=6)	Trained (n=6)	Perfused (n=7)	Trained and Perfused (n=3)
Collagen Content (%)	70.8 ± 0.9	85.0 ± 0.8 *	80.7 ± 1.3	87.3 ± 0.9 *

Discussion/Conclusions:

Self-organized engineered ligament constructs subjected to adult physiologic mechanical training (90° rotation and 10% strain at 0.5Hz for 10min per 24 hours) demonstrated increased percent collagen content relative to controls, which is consistent with previous reports². Ligoids that were perfused without training demonstrated an increased ultimate force. Trained and perfused ligoids demonstrated an increase in diameter and this group also reported a lower ultimate stress than the control group which could be attributed to the significantly increased diameter. All trained ligoids were found to have a significantly higher percent collagen content.

Since other researchers have witnessed mechanical improvements after training in engineered ligament tissue built on exogenous scaffolds³, we speculated that training would convey similar benefits to our self-organized tissue fabrication technique. The size benefit conferred by perfusion with training could assist in an attempt to scale the process to produce clinically significant tissues, however, the current training regime is too intense for our neonatal-like tissues¹. Studies are underway with a lower intensity training regime.

References:

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