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Preliminary study of an *in vitro* development of new tissue applying mechanical stimulation with a bioreactor as an alternative for ligament reconstruction

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ABSTRACT

Complete rupture of the anterior cruciate ligament (ACL) is a common problem in orthopedics. At present, there are many techniques to reconstruct ligaments, which include the use of autografts, allografts, and, in some cases, artificial ligaments. The latter have not provided good results in the short, medium, and long term. The purpose of present study was to engineer functional biological tissue that could potentially be used to replace the knee ligaments by applying tissue engineering techniques and mechanical stimulation with a bioreactor, promoting cellular differentiation and matrix synthesis. In this preliminary study, the new tissue was characterized with mechanical tests and biological tests (viability and immunochemistry), comparing their behavior with that of the native tissue. Mechanical and biological tests proved that mechanical stimulation administered with a bioreactor maintains the ligament fibroblast phenotype and promotes synthesis of the extracellular matrix.

Key words. Anterior cruciate ligament. Bioreactor. Fibroblast. Mechanical stimulation. Tissue engineering. Young's modulus.

Estudio preliminar del desarrollo* in vitro *de un neotejido aplicando estimulación mecánica con un biorreactor como alternativa para la reconstrucción de ligamento

RESUMEN

La ruptura completa del ligamento cruzado anterior (LCA) es un problema común en el área de ortopedia. En la actualidad existen diversas técnicas para la reconstrucción de los ligamentos, las cuales incluyen el uso de autoinjertos, aloinjertos y en algunos casos ligamentos artificiales. Estos últimos no han demostrado buenos resultados a corto, mediano y largo plazo. El propósito del presente estudio es diseñar un tejido funcionalmente biológico capaz de ser usado para el reemplazo de los ligamentos de la rodilla aplicando técnicas de ingeniería de tejidos y estimulación mecánica con un biorreactor, promoviendo la diferenciación celular y la producción de matriz extracelular. En este estudio preliminar, el neotejido fue caracterizado con pruebas mecánicas y pruebas biológicas (viabilidad e inmunohistoquímica), comparando su comportamiento con el del tejido nativo. Las pruebas mecánicas y las pruebas biológicas demostraron que la estimulación mecánica dada por un biorreactor promueve el mantenimiento de la expresión fenotípica de fibroblastos de ligamento y la síntesis de matriz extracelular.

Palabras clave. *Ligamento cruzado anterior. Biorreactor. Fibroblastos. Estimulación mecánica. Ingeniería de tejidos. Módulo de Young.*

INTRODUCTION

Ligaments are parallel bands of dense connective tissue that provide joint stability without limiting motion. One of the most important ligaments in the knee is the anterior cruciate ligament (ACL); this ligament can be easily injured, mainly by trauma factors such as sudden change of direction, fast

decrease of velocity, jumping, or by direct trauma to the joint.[1] According to the American Orthopedic Society for Sports Medicine (AOSSM) in the U.S., 600,000 arthroscopic procedures are performed every year; 85% are performed to treat knee pathology and approximately 125,000 aim to repair or reconstruct the ACL.[2]

The knee ligaments are composed of collagen fibrils (I, III, and V), fibronectin, and tenascin;[3] multiple fibrils produce fibril bundles, and these bundles become a fascicle, which possesses a hierarchical structure. This structure and the combination of the fibrils provide the ligaments with their unique properties and mechanical behavior.[1,4,5]

In order to successfully restore the function of an injured knee, ACL substitutes must offer the same biomechanical behavior as normal ACL (native tissue).

Repair of the ligaments of the knee

For repair or reconstruction of knee ligaments, surgical treatment is necessary; cruciate ligament is not capable of healing or regenerating when a serious injury or rupture occurs.[6]

Recently, knee ligament reconstruction has been performed using tendon autografts or allografts, and even with artificial ligaments; however, the results have not been sufficientlygood.[7,8]

Harvesting of autografts results in potential secondary morbility to the patient because the graft is usually obtained from the hamstring, patellar, or quadriceps tendons. The main benefit of utilizing these grafts is that autologous tissue is well tolerated with no additional adverse reactions. Allografts could potentially transmit bacterial diseases and can result in a sustained inflammatory response. Lack of secondary morbidity at the harvesting site and reduced surgery time are some of the benefits of using allografts.[9] Xenografts have been long since abandoned.

Important efforts have been conducted using artificial ligaments made from a variety of materials such as natural fibers or synthetic polymers, but they have not provided successful results to date. Another option to produce artificial ligaments is the use of degradable and non-degradable fibers, which can be engineered from natural or synthetic polymer-based materials.

Non-degradable materials include carbon fibers, Polyethylene terephthalate (PET), Polypropylene (PP), and Polytetrafluoroethylene (PTFE). Devices made from these materials fail over time because they cannot completely mimic the mechanical behavior of native tissue.

Some natural polymers that are currently in use are based on type I collagen and silk. These materials are biocompatible, bioabsorbable, and biodegradable, and have shown good results in terms of cell attachment, proliferation, and the production of extracellular matrix. The main disadvantage of these is their fiber arrangement. These materials do not offer the appropriate structure; furthermore, they do not prevent long-term failure due to fatigue, creep, and the abrasive wear that is common in the knee.[10,11]

Commonly used synthetic polymers for tissue engineering devices include Polyglycolic acid (PGA), Polylactic acid (PLA), and their copolymers, Polyurethane urea (PUU), Poly desamino tyrosyl-tyrosine ethyl carbonate (poly[DTE]carbonate), Polydioxane (PDS), and Polycaprolactone (PCL). These polymers enjoy the characteristics of being biodegradable, bioabsorbable, and biocompatible; hence, there is no risk of rejection or disease transmission.[12,13] These devices are also designed to be degraded over time, thus avoiding a long-term inflammatory response. The mechanical properties of these devices require control by altering polymer composition, the degree of polymer crystallinity, changing the polymer molecular weight, or changing the ratio of each polymer in the copolymer (Freeman, 2008).

To successfully repair knee ligaments, it would be necessary to have a scaffold with controlled pore size and communication among pores, so that cells can proliferate on it as a three-dimensional (3D) mesh with good distribution of nutrients and resistance to wear and rupture. In this manner, their mechanical properties would be comparable to those of a native ligament such as ACL.[14,15] It has been shown that the application of mechanical stress to fibroblast (ligament cells) can induce differentiation and synthesis of proteins (e.g., collagen types I and III, tenascin, and fibronectin)[16,17] and may change the cell orientation, depending on the stretching of the load.[18,19]

Bioreactors have been used to achieve this goal because they provide an adequate environment for cell culture and mechanical stimulus for these to produce a cellular response.

Bioreactors

The main advantages of a bioreactor are the controlled distribution of the nutrients necessary for

the cells, controlled mass transportation rates, uniform mixing, maintenance of the temperature, pH and partial gas pressures, and controlled shearing stress.[20-23] Some bioreactors can also apply mechanical stimulation to the cells, inducing a cellular response that can be affected by orientation of the load, the tension magnitude, and frequency.[18,24-27]

The aim of this work was to evaluate the possibility of engineering a ligament-like structure by applying mechanical stimulation for ligament fibroblast seeded onto PGA and PLLA scaffolds using a bioreactor with controlled mass transportation rates, uniform mixing, constant temperature, pH, and partial gas pressures under cyclic loads.

MATERIAL AND METHODS

A porous, non-woven PGA/PLLA scaffold was used (BIOFELT®, Concordia Fibers; Concordia Manufacturing Corp., Inc., Coventry, RI, USA) a micrograph of the BIOFELT® is shown in figure 1. The dimensions of the scaffolds employed were 50 mm, 25 mm according to the Standard Test Method (ASTM D3039) (composite tensile modulus measurement) and approximately 0.3–11 mm in thickness according to the manufacturer. The scaffold was sterilized with alcohol 70% and ultraviolet (UV) light radiation. The fibroblasts utilized were harvested from fresh porcine ligaments, by collagenase (Worthing Biochemical Corp., Lakewood, NJ, USA), seeded into 25-cc flasks to expand until second-passage; the medium, changed twice a week, was Dulbecco's modified Eagle's medium (DMEM)/Nutrient mixture F-12, with 10% of Fetal bovine serum (FBS) and 1% Antibiotic antimycotic (AA) at 37 °C and 5% CO_2). Each scaffold was seeded with 12.5 x 106 fibroblasts (1 x 10^6 cells per cm^2). Constructs were incubated in static culture for 24 h, after which they were mechanically stimulated with the Bose ElectroForce® 5200 BioDynamic® Test Instruments 4-chamber Bioreactor from Bose Corp (ElectroForce Systems Group, Eden Prairie, MN, USA) with 4 chambers. The samples were placed into the chambers (one sample per chamber) under sterile conditions and the chambers were filled with DMEM/F-12 with 10% FBS and 1% AA. Once the samples were placed into the chambers with culture medium, the chambers were mounted onto the axial loading frame assembly. Eight samples were studied divided in two groups as follows: the first group was maintained in the bioreactor during 2 weeks, and the second group, for 4 weeks. For each group, one half of the samples received mechanical stimulation. Samples were maintained under controlled conditions (temperature 37 °C, 5% CO_2, and humidity 60%) for the entire period.

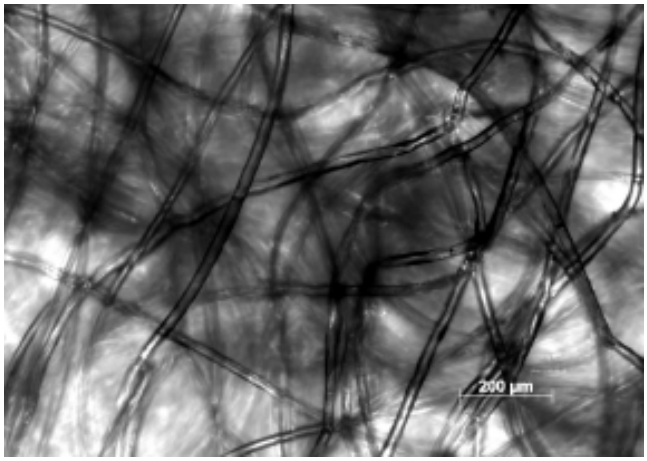

Figure 1. Non-woven PGA/PLLA scaffold (BIOFELT®), without cells and without mechanical stimulation. Scale bar = 200 µm.

Mechanical stimulation

To control the parameters for the mechanical stimulation applied by the bioreactor, we employed

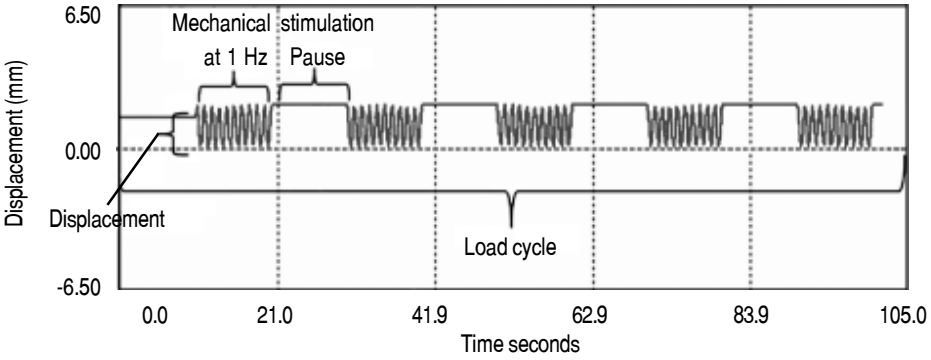

Figure 2. Loading cycle of the mechanical stimulation. At intervals of stimulation and pause.

Win-Test® ver. 4.1 Shareware software. The mechanical stimulation applied to the samples was a continuous repeated cycle for 2 and for 4 weeks. Loading conditions were the following: displacement, 2 mm; pulsatile pump; 2 mL/sec; frequency, and 1 Hz (cadence of the natural human gait). The completed cycle had a block formed by a senoidal wave of 10 cycles at 1 Hz and one of 10 cycles of pause, as depicted in figure 2. This block had a 5-min duration, followed by 5 min of pause, and was repeated for 2 and for 4 weeks.

After the 2 and 4 weeks, the samples were removed from bioreactor under sterile conditions.

Mechanical tests

Samples used for the mechanical tests (fresh porcine ligament; BIOFELT® without cells and without stimulation, and BIOFELT® with cells with and without mechanical stimulation for to 2 and 4 weeks) were fixated with Paraformaldehyde (PFA) 4% (24 h). After fixation, the samples were put into different tubes with Phosphate buffered saline (PBS) solution.

Mechanical tests were performed with a Material Testing System (MTS)® 858 MiniBionix testing machine with a load cell of 111.21 N for the BIOFELT® samples and of 2,000 N for fresh porcine ligaments, at a velocity of 8.3 mm/sec. The distance between clamps was approximately 27.15 mm for the BIOFELT® samples and 20.79 mm for the fresh porcine ligaments, without any pretensioning or conditioning.

The raw data obtained from the tests were processed with MATLAB® software applying a polynomial model to the stress-strain curves to facilitate data handling.

Biological tests

The biological tests were divided into the following two types: the viability tests, and that of immunohistochemistry. Samples for immunohistochemistry (BIOFELT® without cells and without stimulation, and BIOFELT® with cells and with and without mechanical stimulation for 2 and 4 for weeks) were fixated with PFA 1% (30 min). After fixation, the samples were placed into dif-ferent tubes with PBS.

Viability tests were performed with a LIVE/DEAD® Viability/Cytotoxicity Kit (calcein and ethidium homodimer) from Invitrogen Corp (Carlsbad, CA, USA). The culture medium was removed and the samples were washed with PBS, and the calcein and the ethidium homodimer were mixed with culture medium at a concentration of 0.5 μL per milliliter for each reagent. Samples were covered with the reagent and placed in the incubator for 30 min. The reagent was removed and the samples were washed with PBS, following the manufacturer's procedure. Samples were viewed with an inverted fluorescence microscope (Carl Zeiss AxioVert, and AxioVision software; Carl Zeiss, Göttingen, Germany).

Immunohistochemistry was performed to identify type I collagen, fibronectin, and tenascin. The primary antibodies utilized were anti-Collagen type I monoclonal (5D8-G9) from Millipore, Fibronectin (616) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Tenascin-C (cN-19) from Santa Cruz Biotechnology. The secondary antibodies used were fluorescein goat anti-mouse and fluorescein donkey anti goat, both from Santa Cruz Biotechnology. The samples were placed in PBS for 15 min at 4 °C; the PBS was then removed; a blocking solution was

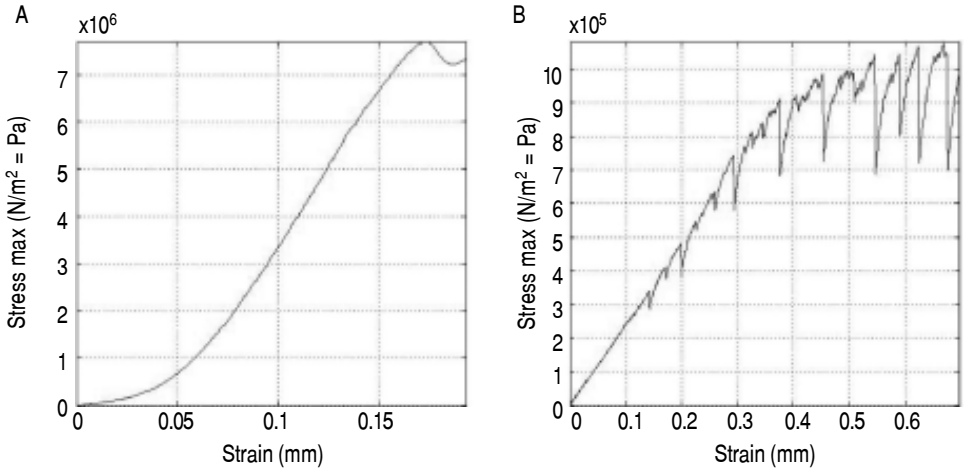

Figure 3. Stress –strain curves without processing (Raw data). A. Porcine ligament. B. Biofelt® without cells and stimulation.

added for 20 min at 4 °C and the blocking solution was then removed. The primary antibodies were added to the samples (one sample per protein) overnight at 4 °C. Samples were washed twice with PBS for 5 min; secondary antibodies were added for 2 h at 37 °C, and then removed; samples were washed with a solution of Triton 0.3%/PBS twice for 5 min each time. Ethidium homodimer was added to the samples for 5 min (0.5%/PBS) and was then removed and washed with PBS. Samples were observed under the microscope to evaluate the presence of the proteins-of-interest in the samples.

RESULTS

The stress-strain curves from the mechanical test are shown in figures 3-9. The results obtained were Young's modulus, stiffness, flexibility, ultimate ten-

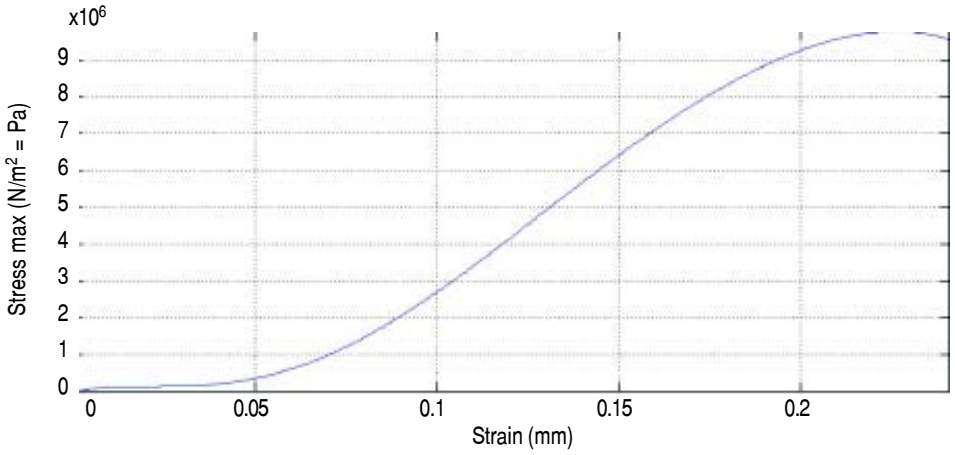

Figure 4. Stress-strain curves from the porcine ligament.

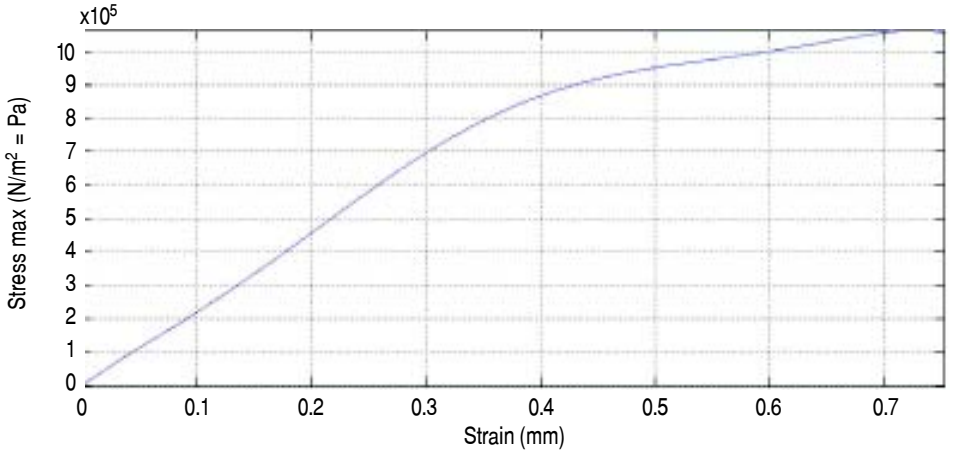

Figure 5. Stress-strain curves from the Biofelt® without cells and stimulation.

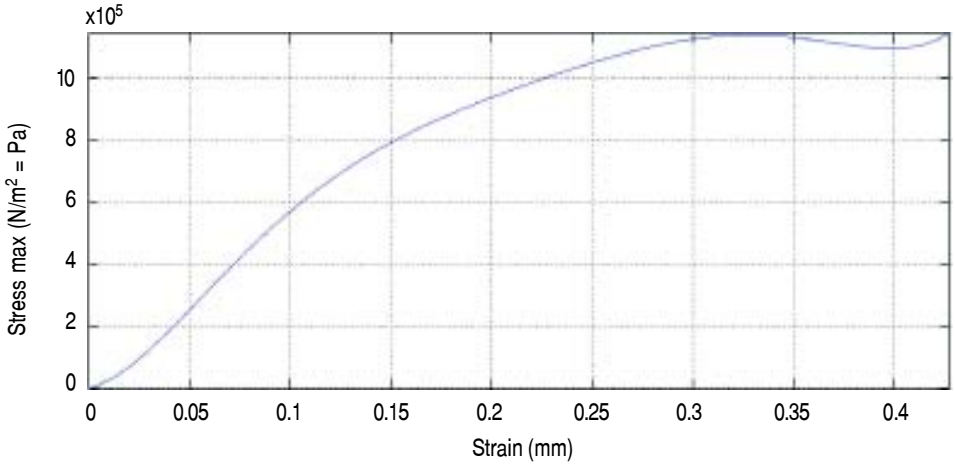

Figure 6. Stress-strain curves from the Biofelt® with fibroblast for 2 weeks without stimulation.

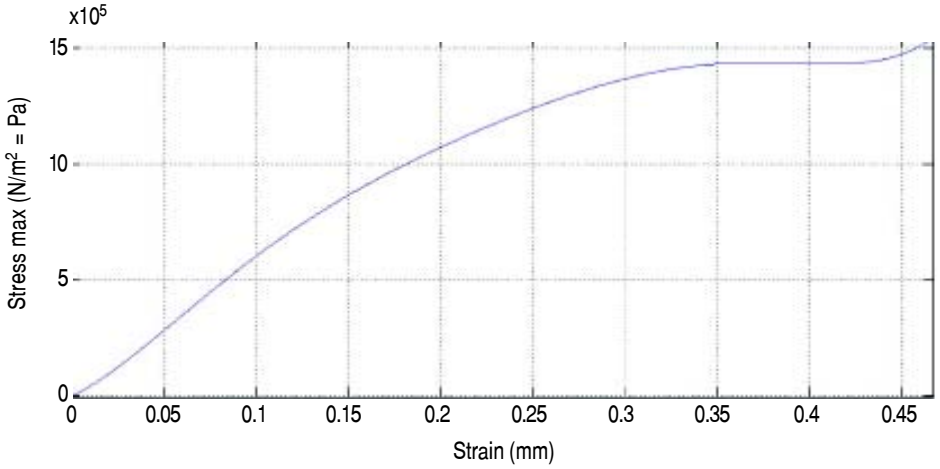

Figure 7. *Stress-strain curves from the Biofelt® with fibroblast for 2 weeks with stimulation.*

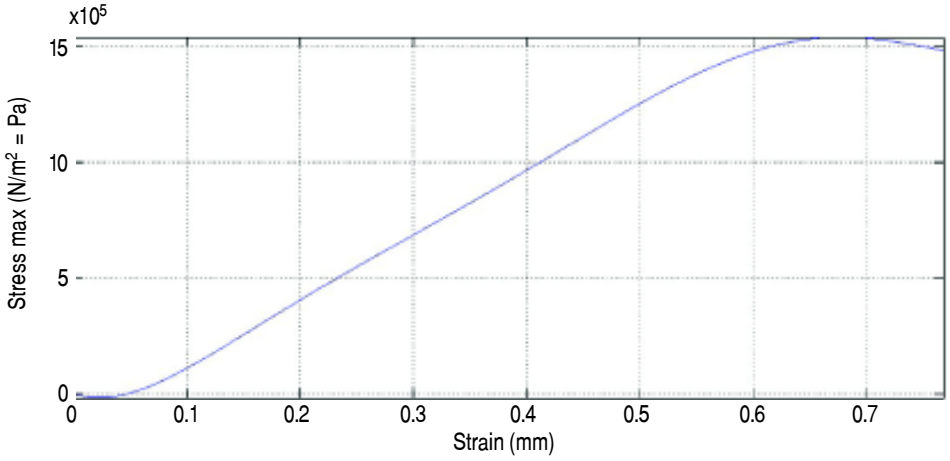

Figure 8. Stress-strain curves from the Biofelt® with fibroblast for 4 weeks without stimulation.

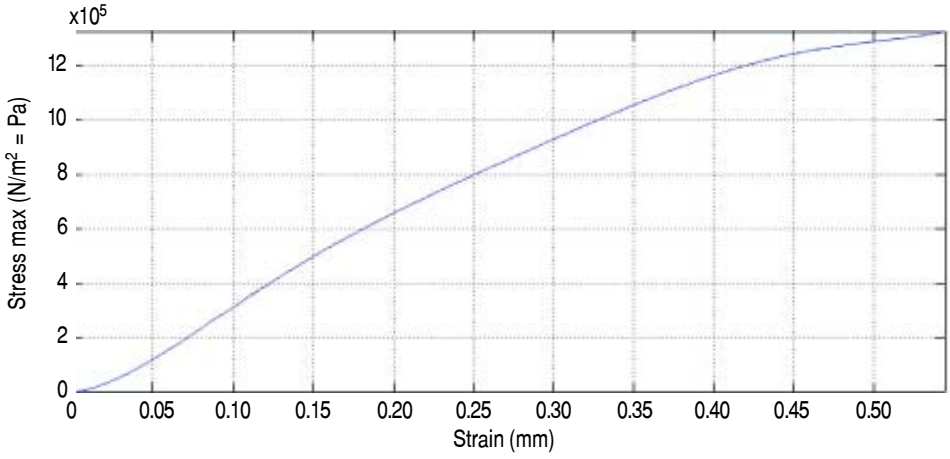

Figure 9. *Stress-strain curves from the Biofelt® with fibroblast for 4 weeks with stimulation.*

sile strength (UTS), maximum deformation, and tenacity, presented in table 1. The results revealed that BIOFELT® does not have the mechanical properties of native ligament.

Viability was observed after seeding ligament cells on to the polymer evaluated, micrographs are shown in figure 10. Immunohistochemistry analysis showed positive stain for type I collagen, tenascin, and fibronectin, proteins characteristic of ligament are depicted in figures 11-15.

DISCUSSION

Stress-strain curves without processing exhibited the mechanical properties of the ligament and of the BIOFELT®; the last one revealed non-smooth behavior,

Table 1. Values of the mechanical tests.

	Fresh Porcine Ligament	Biofelt without cells	Biofelt (2 weeks without stimuli)	Biofelt (2 weeks with stimuli)	Biofelt (4 weeks without stimuli)	Biofelt (4 weeks with stimuli)
Young's Modulus (MPa)	25.80	2.84	5.66	6.36	2.91	3.90
Stiffness (kPa)	55.80	6.93	6.26	7.62	7.11	4.66
Flexibility (μPa)	201	145	160	131	141	214
UTS (MPa)	8.43	1.09	1.28	1.60	1.23	1.44
DefUTS (%)	24.39	34.77	41.18	45.18	71.90	52.89
Maximums deformation (%)	26.38	72.46	42.71	46.70	50.57	54.40
Tenacity (kPa)	70.70	46.20	33.40	45.50	51.70	40.90

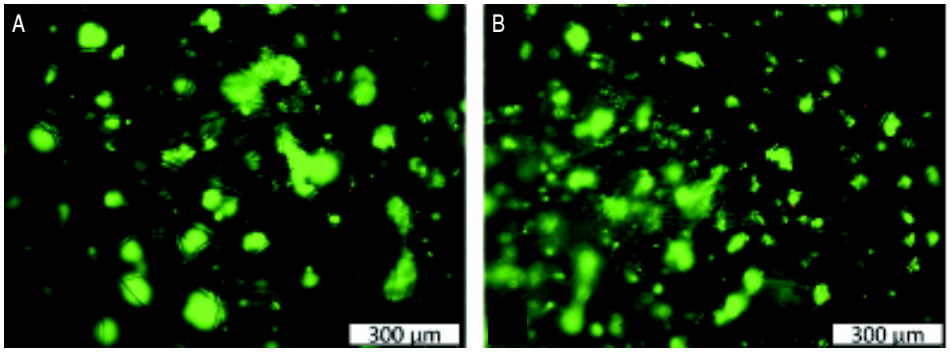

Figure 10. *Viability test. 2 weeks A. Without stimulation. B. With stimulation. Scale bars = 300 μm.*

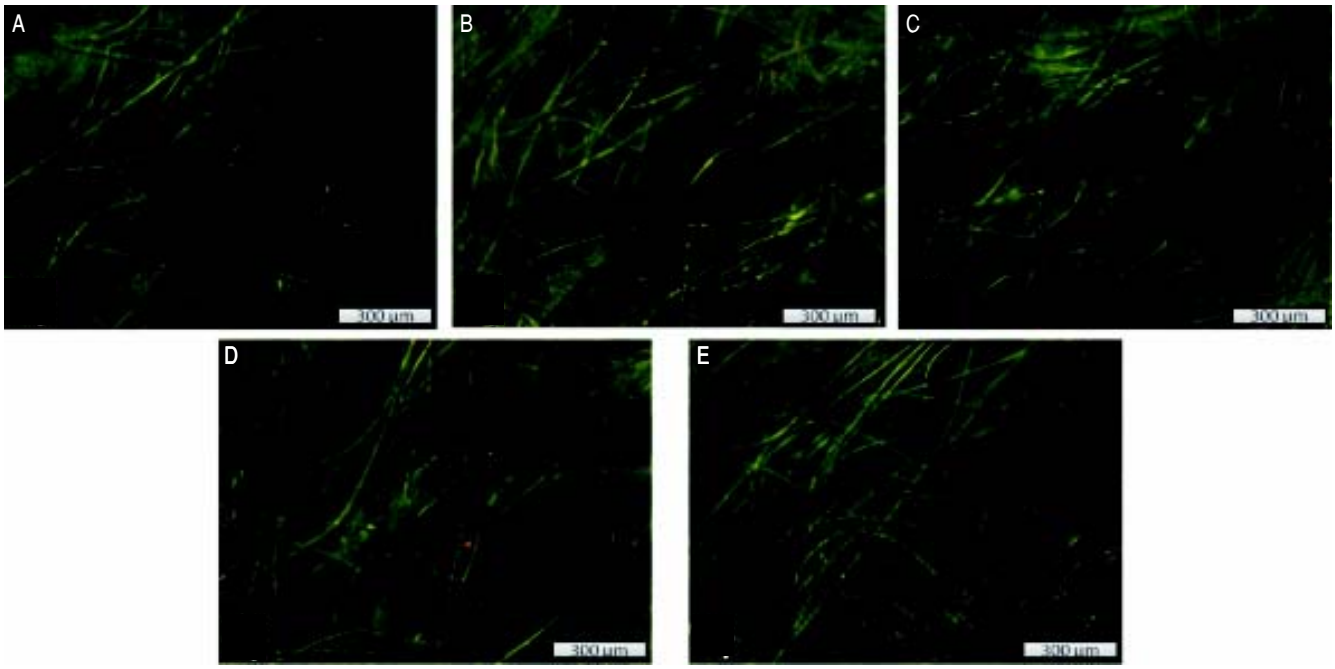

Figure 11. Immunohistochemistry tests. Biofelt® without cells and without stimulation. A. Type I Collagen. B.Tenascin. C. Fibronectin. D. Mousse control (Type I collagen and Tenascin). E. Goat control (Fibronectin). Scale bars = 300 μm.

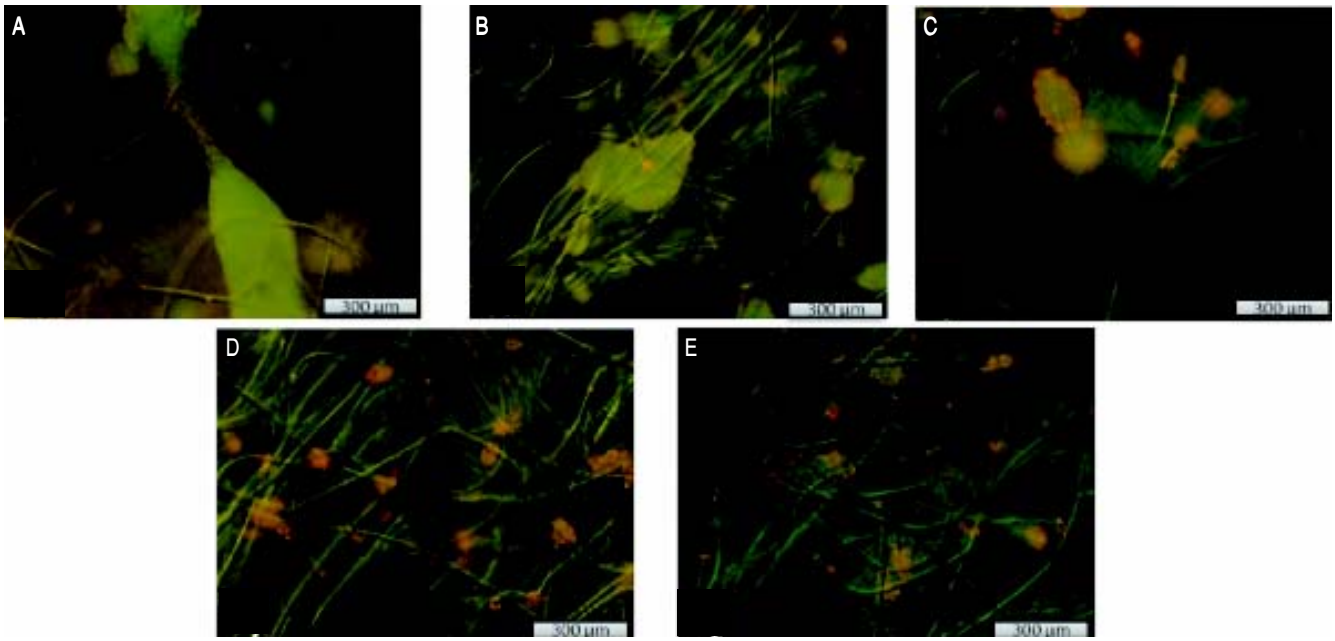

Figure 12. *Immunohistochemistry tests. Biofelt® with fibroblast, 2 weeks without stimulation.* ***A.*** *Type I Collagen.* ***B.****Tenascin.* ***C.*** *Fibronectin. D. Mousse control (Type I collagen and Tenascin). E. Goat control (Fibronectin). Scale bars = 300 μm.*

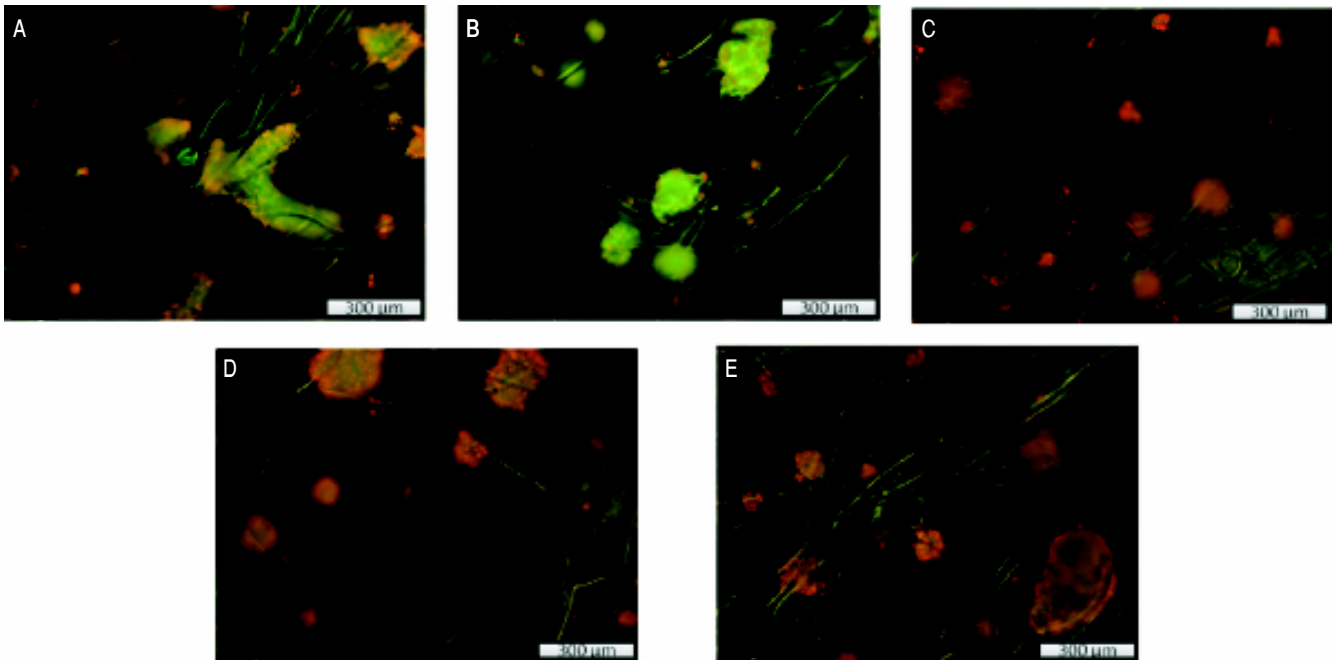

Figure 13. Immunohistochemistry tests. Biofelt® with fibroblast, 2 weeks with stimulation. A. Type I Collagen. B.Tenascin. C. Fibronectin. ***D.*** *Mousse control (Type I collagen and Tenascin).* ***E.*** *Goat control (Fibronectin). Scale bars = 300 μm.*

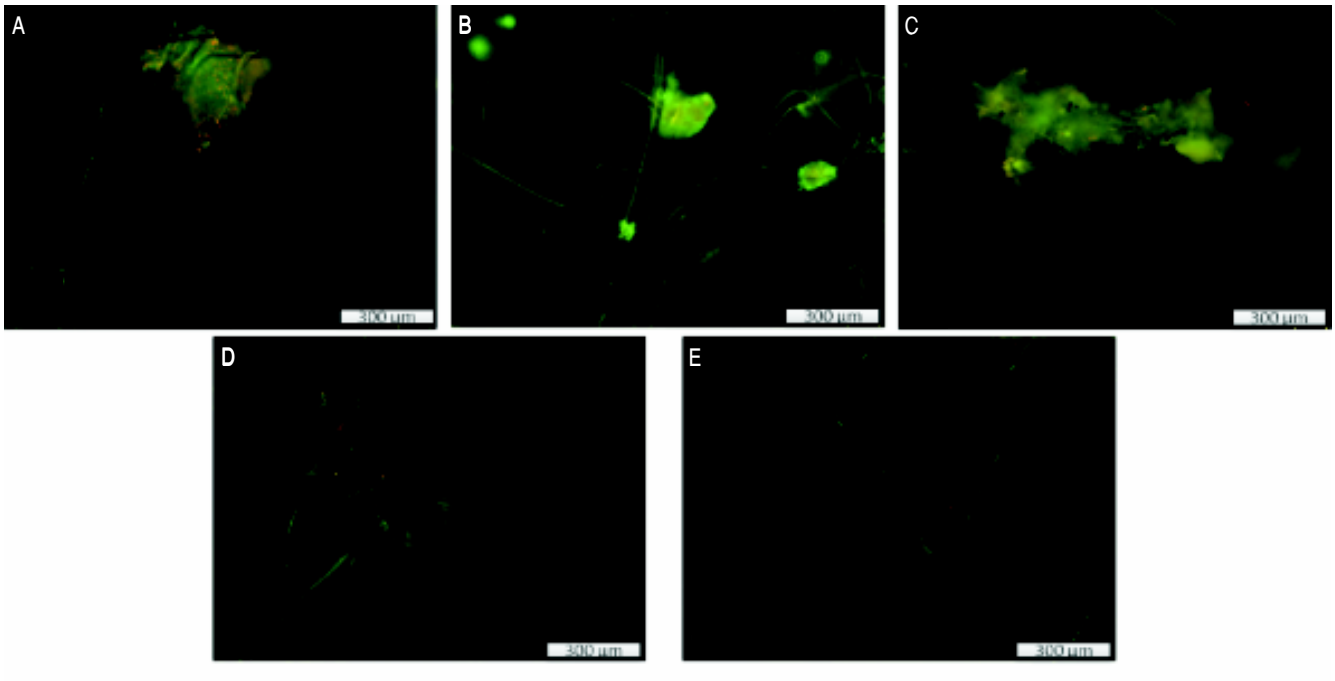

Figure 14. Immunohistochemistry tests. Biofelt® with fibroblast, 4 weeks without stimulation. A. Type I Collagen. B.Tenascin. C. Fibronectin. D. Mousse control (Type I collagen and Tenascin). E. Goat control (Fibronectin). Scale bars = 300 μm.

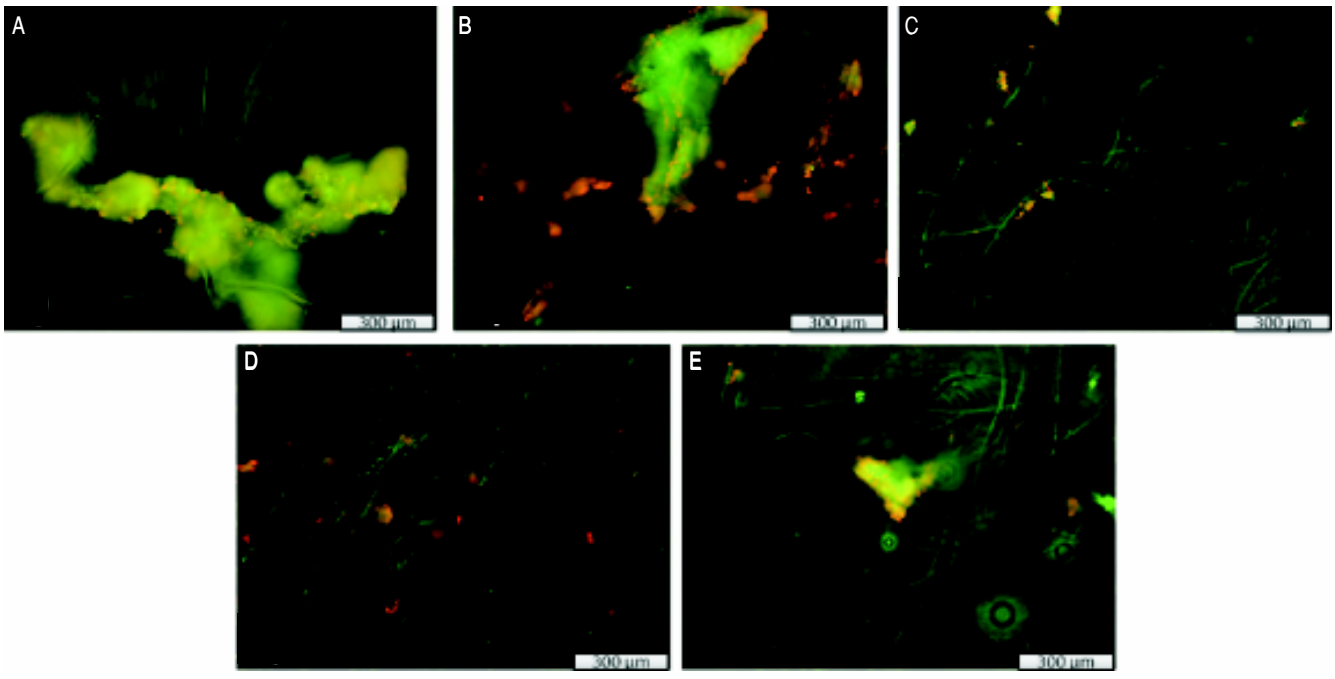

Figure 15. *Immunohistochemistry tests. Biofelt® with fibroblast, 4 weeks with stimulation.* ***A.*** *Type I Collagen.* ***B.****Tenascin.* ***C.*** *Fibronectin. D. Mousse control (Type I collagen and Tenascin). E. Goat control (Fibronectin). Scale bars = 300 μm.*

due to random fibrils than conform the non-woven scaffold, giving it the pattern shown in figure 3B, in comparison with the ligament, which has unidirectional fibers.

Mechanical tests revealed that the BIOFELT® does not resemble the characteristics of native tissue (ligament), but when cells and stimulation are applied to the polymer, its behavior is modified and it resembles the one of native tissue. BIOFELT® is easily deformed; Young's modulus from native tissue is 25.80 MPa, the BIOFELT® without cells has a Young's modulus of 2.84 MPa, BIOFELT® with fibroblast for 2 weeks without stimulation has a Young's modulus of 5.66 MPa, with stimulation 6.36 MPa, and BIOFELT® with fibroblast for 4 weeks without stimulation has a Young's modulus of 2.91 MPa and with stimulation 3.90 MPa. These results demonstrate that native ligament requires much additional strain in order to deform than BIOFELT®; this value is increased when the cells are seeded and it increases to an even greater degree when stimulation is applied. This occurs in polymers at 2 and 4 weeks; however, results at 2 weeks are much closer to those of native tissue behavior than the results at 4 weeks. This might be due to the BIOFELT® degradation. This tendency is also shown for stiffness, tenacity, and UTS (as well as for defUTS, which indicates de UTS in terms of percentage). Stiffness, tenacity, and UTS are related parameters. Stiffness, as well as tenacity, reflect difficulty in breaking the native tissue (55.80 kPa) in comparison with the BIOFELT® (69.30 kPa); in addition, the UTS informs us that it is necessary to apply much force to make the ligament significantly lose part of transversal section (84.30 kPa), compared with the BIOFELT® (1.09 kPa). Native tissue has a flexibility value of 201 μPa; this value is comparable in the polymers, which have the following: BIOFELT® 145 μPa, BIOFELT® with fibroblast without stimulation for 2 weeks, 160 μPa; BIOFELT® with fibroblast with stimulation for 2 weeks, 131 μPa; BIOFELT® with fibroblast without stimulation for 4 weeks, 141 μPa, and BIOFELT® with fibroblast with stimulation for 4 weeks, 131 μPa; these values do not possess an apparent pattern, but samples at 4 weeks with stimulation possess behavior closer to that of the ligament. Maximal deformation revealed how difficult it is to deform native tissue (26.38%) in comparison with BIOFELT® samples, which are exceedingly easy to deform, particularly BIOFELT® without cells (72.46%).

On the other hand, micrographs from the viability test revealed that BIOFELT® allows attachment and cell proliferation during this time. Immunohistochemistry identifies type I collagen, fibronectin, and tenascin proteins, which are the principal proteins deriving from the extracellular fibroblast ligament matrix. Micrographs of BIOFELT® without cells are used for control, because the material possesses its own fluorescence. Type I collagen appeared in all of the samples, at 2 or at 4 weeks, with or without stimulation; thus, the test was positive for this protein. Fibronectin appears in all of the samples, but is much more marked in samples with stimulation at 2 and at 4 weeks; thus, the test was positive for fibronectin. Tenascin appears in all of the samples, to a lesser degree compared with the other proteins. This protein also follows the same pattern as the fibronectin, which appears much more marked in samples with stimulation; the test was positive for the tenascin.

Therefore, we can conclude in this preliminary study that mechanical stimulation is a crucial factor to maintain ligament fibroblast phenotype, because samples with stimulation proved to have many more proteins in extracellular matrix than samples without stimulation. Mechanical tests demonstrated that BIOFELT® with this structure is not functional for replacing the ligament, because it does not comply with the mechanical characteristics necessary to supply it, but it is benefited when the cells are seeded and stimulation is administered.

Limitations of the study

Although the study shows that the use of mechanical stimulation with a bioreactor can lead to the maintenance of ligament fibroblast phenotype and matrix synthesis, the scaffold employed did not comply with the mechanical properties of native ligament. The utilization of a knitted scaffold with a different structure should be addressed to solve this problem in future studies.

A larger sample is also recommended in order to conduct a quantitative study in the future. However, this preliminary study exhibited the benefits of the mechanical stimulation provided by a bioreactor.

ACKNOWLEDGMENTS

This work is part of project sponsored by the Sectorial Fund for Health Research and Social Security: Key CONACyT-SALUD-2007-01-71011.

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Recibido el 02 de septiembre 2013.
Aceptado el 03 de octubre 2013.