Monitoring mechanical stimulation for optimal tendon tissue engineering: a mechanical and biological multiscale study

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ABSTRACT

To understand the effect of mechanical stimulation on cell response, bone marrow stromal cells were cultured on electrospun scaffolds under two distinct mechanical conditions (static and dynamic). Comparison between initial and final cell construct mechanical and biological properties were conducted over 14 days for both culturing conditions. As a result, mechanically stimulated constructs, in contrast to their static counterparts, showed evident mechanical-induced cell orientation, a high amount of aligned collagen along with an abundance of tenomodulin differentiation phenotype. Cell orientation and high collagen production and orientation lead to enhanced storage modulus observed under dynamic stimulation. The increase in tenomodulin under dynamic conditions provided clues on the importance of mechanical stimulation to induce tendon-like phenotype production. Altogether, mechanical stimulation lead to (i) morphological orientation were static cultured constructs presents random morphology, (ii) more pronounced elastic behavior with a limited viscous contribution in the global mechanical response. Such a correlation could help in further studies to use mechanical stimulation as chemical-free cell guidance in tissue engineering development.

Key words: Electrospinning, Mechanical stimulation, Stem cells, Tissue engineering, tendon

Introduction

The main objective of tendon tissue engineering is to design and produce a cell-construct that will help to regenerate damaged tissue or mimic it for comprehensive in vitro studies. To achieve this goal, different groups have searched in recent years for the most efficient cells, the right materials, and the appropriate chemical or mechanical environment(1,2). Although the holy grail is still a long way off, most studies have clearly demonstrated the advantages of reproducing the mechanical environment in order to guide the cell-scaffold constructs towards tendon repair(2).

Biomimetic analysis can be considered as essential to guide choices in advanced tissue engineering. Native tendon is made of an anisotropic and viscoelastic material capable of resisting high tensile forces. Tendon cells, mainly tenocytes, are rather scarce, and are responsible for synthesising extracellular matrix, which is mainly composed of type I collagen with a highly organised structure.

As primary tenocytes are rather difficult to collect, bone marrow stromal cells (BMSC) are the most widely used stem cells for tendon tissue engineering, among other stem or progenitor cells, as they have the potential for self-renewal, clonogenicity, and multi-lineage differentiation, including tenogenicity(3,4).

Regarding the scaffold issue, electrospinning has been used for several years to tailor an environment for cell development and differentiation similar to that of extracellular matrix, but with different fibre sizes, porosity, elasticity and mechanical properties for tendon tissue engineering(5). Recently, Lee et al. showed that small, nano-scale random fibres provided a cell environment similar to that found in the inflammatory phase of the tendon healing process, promoting the synthesis of the extracellular matrix (ECM) and cell proliferation, while larger aligned fibres mimicked the normal structure of collagen in tendon, maintaining the tendon cell phenotype(6).

Mechanical stimulation is another key environmental factor for reproducing in vivo conditions. Physiotherapists recommend periodic stretching in training to heal defects or to

improve capacity(7–10). In vitro, some studies have stated that proper stimuli applied to biohybrid scaffolds could act on cell proliferation, differentiation or function, following mechanotransduction pathways(11–13). While different stimuli have been tested with a wide range of amplitudes, frequencies and time, it should be noted that little is known about how mechanical stress may affect both cell and material responses throughout the tissue engineering process(14). In addition, the parameters assessing the evolution over time of the cell-constructs' mechanical properties are not always relevant. Elastic modulus, stiffness and ultimate tensile strength seem to be accepted as the standard in the field(4). However, native tendon is a viscoelastic material, combining viscous liquid-like, and solid-like behaviour. While the notion of viscoelasticity includes time dependency, meaning that the mechanical response depends on the deformation rate (ε), tendon tissue engineered constructs tend to be characterised by quasi-static mechanical tests as ranges of strain that do not reproduce physiological conditions. These parameters emphasise on the elastic properties of the material, at a supra-physiological level and with a limited real application. Measured at the beginning and at the end of each experiment, a day-to-day evolution of the mechanical properties of different materials remains utopic.

The objective of this study was to establish a time resolving monitoring approach of the mechanical properties of cell construct under different culturing conditions. Thus, the precise and relevant change in the measured mechanical properties could be used to interpret the cells' responses in close correlation to the chosen biological indicators. Such correlation could help in further studies to use mechanical stimulation as chemical-free cell guidance in tissue engineering development.

In order to better understand this inter-dependency between mechanical stimulations and biohybrid scaffold responses, we performed static and dynamic cultures of rat BMSCs on dedicated random electrospun PCL scaffolds using bioreactors (T6 CellScale and Bose Biodynamic 5100). The mechanical behaviour under a pseudo-physiological strain and cellular activity of the cell-constructs during the stimulation period were recorded and analysed for 12 days, then compared with those obtained under static conditions. At the biological level, we focused on cell proliferation, differentiation towards tendon lineage (in the absence of specific differentiation factors) and organisation of the neo-synthetized ECM.

At the mechanical level, we followed up the changes in both the viscous and elastic properties of the pure and cell-seeded scaffolds. Therefore, we propose to evaluate different mechanical markers of both elasticity and viscoelasticity such as energy dissipation, damping factor (tan δ), storage modulus (E') and loss modulus (E"), already used in the mechanobiology field to analyse the performance of native tendons.

Materials and Methods

- **Electrospun scaffold preparation and characterisation**

Poly(ε-caprolactone) (PCL, MW=80kg.mol^{-1} Sigma Aldrich, United States) was dissolved in dichloromethane (DCM, Sigma-Aldrich)/*N,N*-dimethylformamide (DMF, ReagentPlus® Sigma-Aldrich, USA) (80/20 v/v) for 24h to make an electrospinning solution at 10 wt %. Once dissolved, the solution was poured into a 10 ml glass syringe. Scaffold fabrication was performed over a rotating collector for 3H (distance 15 cm, flow rate 0.017ml/min, needle diameter 18G, voltage 15kV). In order to evaluate the morphology and mechanical properties of the PCL scaffolds, scanning electron microscopy and tensile testing were carried out retrospectively.

The morphology of the electrospun scaffolds was observed using scanning electron microscopy (Philips XL30 ESEM-FEG). Electrospun mats were cleaned with ethanol and gold-coated prior to observation. To analyse the diameter of the electrospun 10 wt % PCL fibres, ImageJ software was used. After setting up the scale, a line was drawn manually across the diameter of randomly-selected fibres (n=50) from 3 different SEM micrographs. The degree of isotropy in two samples from three scaffolds (n=6) was analysed using Mountain™ software. The main directions of the fibres were analysed using the Fourier Transform method.

The scaffold modulus for dry and wet scaffolds was quantified using uniaxial tensile testing. Three samples for each scaffold (n=3) were shaped into a strip measuring 1.0 cm x 3.0 cm. For wet samples, the scaffolds were immersed in ethanol 70% (Sigma-Aldrich, USA) for 45 min then washed three times with PBS 7.4 (phosphate buffered saline, Gibco Invitrogen, USA). The thickness was evaluated using a precision dial thickness gauge (Mitutoyo Corporation, Japan). The samples were secured within the metallic grips of the tensile tester

(Bose Electroforce 3200, TA, USA) and elongation at 0.1 mm s^{-1} was performed with a working load of 22N. The applied force was measured each second and the modulus obtained from the slope of the linear region.

- **Cell harvesting and culture**

Bone marrow stromal cells (BMSCs) were isolated from rat bone marrow thanks to their short-time adherence to plastic, in accordance with previously described protocols(15). Briefly, 6-week-old male Sprague Dawley rats (n=4) were sacrificed, and both right and left femurs were aseptically removed and washed 3 times with 1x PBS 7.4 (phosphate buffered saline, Gibco Invitrogen, USA). Next, bone marrow was flushed out using α-MEM culture medium (PAN BIOTECH, Germany) supplemented with 10% foetal bovine serum (FBS, Gibco Invitrogen, USA), 1% penicillin-streptomycin (Gibco Invitrogen, USA) and 1% amphotericin B (PAN BIOTECH, Germany). The released cells were then collected into 6-well dishes (BD Falcon™, USA). After 24h, non-adherent cells were carefully discarded and adherent cells were cultured with fresh α-MEM for 6-7 days, the time needed for BMSC colonies to reach confluence. The cell culture media were replaced every 3 days. When the culture dishes started to approach confluence, the cells were detached and serially subcultured. The cells at the third passage (P3) were used for the cell seeding experiments. Experimental procedures involving animals were carried out on euthanised animals within the approved structure (E60 159 01). Both the procedures and the treatment of the animals complied with the principles and guidelines of the French legislation on animal welfare (No. 2013-118) and the Directive if the European Communities (2010/63/EU).

Electrospun mats were cut into strips measuring 40 x 12.5 mm or 35 x 9 mm as shown in **Supplementary Table ST2**, disinfected with ethanol 70% (Sigma-Aldrich, USA) for 45 min, and then washed three times with PBS for 10 min. Disinfected scaffolds were soaked in fresh α-MEM for 48h before seeding the cells. After that, the media was discarded and each scaffold was plated with a density of $6x10^4$ cells cm^{-2}.

- **Mechanical stimulation**

After two days of static culture, each construct was placed in a bioreactor for mechanical stimulation or in well dishes for static culture. Mechanically-stimulated cell constructs were stretched twice a day at 5% strain for 1h at 1Hz with 11h of rest between each cycle for 5 or

12 days (considered as 1 or 2 weeks of culture time respectively). For this purpose, two different bioreactors were used: (1) the MechanoCulture T6 Mechanical Stimulation System (CellScale Biomaterials Testing, Waterloo, ON, Canada), consisting of an actuator and screw-driven clamp grips mounted inside a cell culture chamber capable of applying uniaxial stretching to 6 parallel samples, was chosen for the biological assays. Cell culture media were replaced every 5 days, and (2) the Bose Biodynamic 5100 (TA Electroforce®, USA), consisting of a cell culture chamber connected to a flow pump in which one sample was attached thanks to a system of rods and clamps. One rod was attached to a step motor making it possible to apply uniaxial displacement. The other rod was connected to a force transducer of 22N making it possible to constantly monitor the force applied to each displacement. Cell culture media circulated continuously through the flow pump connected to a reservoir of 500 mL, making it possible to maintain the culture for up to two weeks. This system was chosen for biomechanical evaluation. For static culture, each construct was cultured for 5 or 12 days without tension and the cell culture media were replaced every 3 days.

Biological and mechanical evaluation of cell-constructs

To investigate the effects of dynamic culture on cell activity, seeded PCL 10-wt % scaffolds were secured in the grips of the T6 CellScale bioreactor after two days of static culture and cultured for 5 or 12 days under dynamic culture conditions (5% strain for 1h at 1Hz with 11h of rest). After this time, the cell-constructs were removed for biological analyses.

Total deoxyribonucleic acid (DNA) and collagen from each sample were extracted at 5 or 12 days of static or dynamic culture with the reagent, Trizol (TRI Reagent®, Sigma-Aldrich, USA) according to the manufacturer´s protocol. Briefly, once lysed by the action of 1ml of Trizol, chloroform (Sigma-Aldrich, USA) was added to obtain a colourless upper aqueous phase with RNA, an interphase with DNA, and a lower red phenol-chloroform phase with proteins. DNA was then isolated and quantified using NanoDrop ND-1000 (Thermo Scientific, USA). Proteins were isolated and hydrolysed in 6N HCl (Sigma-Aldrich, USA), and total hydroxyproline content was determined using hydroxyproline assay (Hydroxyproline Assay Kit, Sigma-Aldrich, USA)(16). Hydroxyproline content was related to the collagen content(17).

After 5 or 12 days of static or dynamic culture, cells-constructs were fixed in a solution of 4% (w/v) paraformaldehyde solution (PAF, Agar Scientific, United Kingdom) in PBS for 15 min then rinsed three times with PBS 7.4. After 10 min of permeabilization in a solution of PBS-Triton X-100 0.5% (v/v) (VWR, United Kingdom), cell-constructs were blocked at 4ºC overnight with a solution of 1% (w/v) BSA (Sigma-Aldrich, USA). The morphology of the rBMSCs under static or dynamic culture was assessed using rhodamine phalloidin (Invitrogen, USA) to selectively stain the F-actin. For immunofluorescence staining, cell-constructs were treated with mouse primary antibodies anti-rat collagen type I (COL1, 1:100, Abcam, United Kingdom) or to anti-rat tenomodulin (TNMD, 1:200, Abcam, United Kingdom) overnight at 4ºC. After incubating overnight at 4ºC with secondary fluorescent antibodies donkey anti-mouse 488 (Invitrogen, USA). Hoechst 33342 (Sigma-Aldrich, USA) was added as counterstaining for cell nuclei. Z-stacks were then acquired on an Inverted ZEISS 710 confocal microscope (Zeiss, Germany).

To investigate the effects of dynamic culture on the evolution of the mechanical properties of the cell-constructs, seeded scaffolds or scaffolds without cells were mounted in the Bose Biodynamic 5100 for 12 days under mechanical stimulation. Bose Biodynamic 5100 consists of one culture chamber with two rods, one connected to a motor and the other to a force transducer of 22N. The entire system is placed in an incubator making it possible to control the temperature and $C0_2$ at 37ºC and 5% respectively. Cell culture media circulated continuously thanks to a system of peristaltic pumps connected to the cell chamber. The bioreactor makes it possible to apply defined cyclic (sinusoidal) strain to the cell-seeded or empty scaffolds, and to concomitantly record the resulting stress. Either stress (σ) or strain (ε) could be set up as the driving parameter. We decided to set deformation, 5% cyclic sinusoidal strain as the control parameter. We recorded the resulting force at 20 points per second, corresponding to 20 points per sinus, during the 12 days of dynamic culture.

$$Strain\ \varepsilon = \varepsilon_0 \sin wt\ (1)$$

$$Stress\ \sigma = \sigma_0 \sin\ (wt - \delta)(2)$$

Where ε_0 and σ_0 are the initial strain and stress respectively, and ω and δ are the frequency and dephasing angle between stress and strain respectively. Plotting together stress vs strain, we were able to calculate the relative dissipation energy by calculating the surface

between the curves in each sinus. Because sinusoidal stress was applied, we also determined the storage modulus (E'), loss modulus (E''), complex modulus (E*) and tan δ. All equations are indicated below:

$$E' = \frac{\sigma_0}{\varepsilon_0}\cos(\delta) \quad (3)$$

$$E'' = \frac{\sigma_0}{\varepsilon_0}\sin(\delta) \quad (4)$$

$$E^* = \sqrt{E'^2 + E''^2} \quad (5)$$

$$\tan(\delta) = \frac{E''}{E'} \quad (6)$$

For analysis, each 1h cycle, consisting of 3600 sinuses, was divided into six intervals of 600 sinus and the results are given as an arithmetic average. After the first analysis, we decided to represent the results from 1200 to 3600 sinus of each cycle, where a closed loop region was found. For statically-cultured cell-constructs and controls, each sample was placed on the bioreactor and, once secured between the rods, one cycle of 3600 sinus was set at a given time (7 and 14 days) to compare in the same way as the dynamic conditions. For the control without cells, scaffolds were subjected to the same parameters as the cell-constructs, including disinfestations and incubation with cell culture media.

Statistical analysis

At least 6 independent experiments, except for the biomechanical analysis (n=3) carried out on the Bose Biodynamic 5100, were performed. Each result (mechanical properties as damping factor, loss and storage modulus) represents an arithmetic average the different analyzed tests. Data are therefore presented as an average along with the appropriate mean standard deviation. The significance of the results was tested by analysing the variance (ANOVA) with Turkey´s post hoc test in the case of multiple comparisons.

On behalf of the authors, we declare, that all the methods were carried out in accordance with relevant guidelines and regulations. In addition all experimental protocols were approved by our institution (Sorbonne Université Alliance, Université de Technologie de Compiègne) commitees.

Results

- **PCL scaffold synthesis and characterisation**

Scaffolds were prepared using electrospinning laboratory made setup **(Fig 1).** The prepared scaffolds were meant to be used for cell culture under different conditions; static and dynamic culturing as presented schematically in figure 2 (**Fig 2**). Electrospinning PCL into a DCM/DMF 4:1 co-solvent led to the production of a homogenous scaffold without pearls. SEM images showed a final material composed of a dense network of continuous smooth fibres **(Fig 3A),** as found in (18). These fibres presented an average diameter of 0.52 ± 0.25 µm. Distribution analyses of the fibres revealed a random conformation **(Fig 3B)**.

The scaffolds were then characterised following the uniaxial traction test described in the Materials and Methods section. In order to evaluate potential alteration of the mats in an aqueous solution, the scaffolds were analysed in both dry and wet conditions. Stress/strain profiles were similar, showing a "J" zone, characteristic of the nonlinear mechanical response of material, in which we were able to identify three different regions; the toe region (<2% strain), heel region (<8% strain) and linear region (<18% strain) **(Fig 4A)**. Both elastic moduli were similar, with 8.05 ± 0.82 MPa for the dry scaffolds and 7.93 ± 2.66 MPa for the wet ones **(Fig 4B).**

- **Biological outcomes and tendon extracellular matrix deposition**

The number of cells presented on the constructs was assessed by means of DNA quantification. The amount of DNA was observed in either static or dynamic conditions after 2 weeks **(Fig 5#)**. Dynamic stimulated cell-constructs appeared to have greater number of cells compared to static cultures with 4.1 ± 1.3 x 10^6 vs 2.91 ± 0.8 x 10^6 respectively, after 2 weeks of culture, although the difference was not statistically significant.

In order to determine the effect of the mechanical stretching on ECM neo-synthesis, hydroxyproline was measured **(Fig 5§)**. Hydroxyproline concentration is related to fibrillar collagen and comprises around 13.5% of the collagen(19). It could be detected in all the culture conditions. After a week of culture, no difference in collagen production was observed between the static or dynamic conditions. After two weeks, a significant increase in collagen synthesis under dynamic stretching was noted, with 18.8 ± 2.6 µg vs 12.1 ± 1.4

µg of hydroxyproline for the static culture. Mechanical stimulation thus induced elevated collagen content in the scaffold, compared to the static culture.

To better understand cell organisation, fluorostaining of the actin cytoskeleton was performed **(Fig 5*)**. Cells cultured in the absence of mechanical stimulation presented a random morphology on the scaffold. This behaviour did not evolve with culture time, with the same observations at 1 or 2 weeks **(Fig 5* A-D).** On the contrary, when submitted to mechanical stimulation, cells presented an elongated shape and appeared aligned with the stretching direction. This effect seemed more pronounced after two weeks of stimulation, with thinner elongated cells at the surface of the material **(Fig 5* D).**

To determine the possible differentiation of BMSCs towards tendon lineage under both static and dynamic culture conditions, immunofluorescence staining of type 1 collagen, the main constituent of tendon ECM, and tenomodulin, a tendon specific marker, was performed at different time points**.** Type-1 collagen was found in both conditions at 1 or 2 weeks of culture **(Fig 5* E-H)**. After 1 week of culture, type-1 collagen structures were aligned with the stretching under dynamic conditions **(Fig 5* F)**, while in static culture they were present in a random manner **(Fig 5* E).** After 2 weeks of culture, the same trend was confirmed. Collagen fibres seemed to be more abundant compared with the first week of culture **(Fig 5* G, H)**. While there were some clusters of aligned collagen on the static culture **(Fig 5* G)**, under the effect of mechanical stimulation, the collagen was highly organised, with collagen fibres aligned towards the stretching axis.

Immunofluorescence staining revealed the presence of tenomodulin on both culture conditions. In the same manner as collagen fibres, an alignment was observed when the cell-constructs where mechanically stretched **(Fig 5* I-L)**. After one week of culture, tenomodulin appeared to be clearly aligned under dynamic conditions **(Fig 5* J)** compared to the static culture **(Fig 5* I)**. This effect was more difficult to visualise after two weeks due to the total distribution of tenomodulin and the high cell density **(Fig 5* K, L)**.

- **Mechanical stimulation and biomechanical evaluation of cell construct**

Initially, static tests were conducted on dry and wet scaffolds. Those tests help deriving elastic modulus (E) by plotting the slope of the stress vs strain curve, corresponding to the linear zone of our material as shown in figure 4 **(Figure 4),** due to the non-linearity at the

initial part of the curve. Dynamic testing were, however conducted at 5% dynamic strain, which means that the dynamic strain was imposed within the non-linear regime of the stress-strain curve.

After 2 days of static culture, the cell-construct or the control scaffolds were fixed inside the bioreactor chamber and the mechanical test was launched. Both signal force and displacement were recorded over time and found to be smooth, without any background noise, making it possible to monitor mechanical (elastic and viscoelastic) properties **(Fig 6A)**.

The relative dissipation energy or hysteresis can be calculated by calculating the surface area between the curves, along with the damping factor **(Fig 6A).** All the relevant parameters were extracted following the equations described previously and presented in **Fig 7**, to follow the tissue response continuously instantaneously and after two weeks of culture.

Initially (i.e. on day 2), in terms of viscous (tan δ, dissipated energy and E´´) and elastic (E´) properties, along with the complex modulus (E*), no **significant differences** were found between the different parameters for the different culture conditions with or without cells, however, slight trends can be drawn between the different conditions. For tan δ, similar values were found for each condition in both cell constructs and controls with 0.08 ± 0.01 vs 0.12 ± 0.03 in static conditions and 0.09 ± 0.01 vs 0.09 ± 0.03 in dynamic ones. For the dissipated energy (hysteresis), cell-constructs seemed to present lower values than controls, with 0.64 ± 0.35 vs 0.92 ± 0.14 in static conditions and 0.90 ± 0.11 vs 1.47 ± 0.57 in dynamic ones **(Fig 7)**. For E'', another parameter related to viscosity, the same trend was found, with lower values for cell-constructs (0.30 ± 0.16MPa & 0.37 ± 0.13MPa) in both static and dynamic conditions, compared to controls (0.42 ± 0.07MPa & 0.59 ± 0.14MPa). In terms of elastic properties, the storage modulus (E') appeared higher for the dynamic control with 6.63 ± 0.89 MPa. E* followed the same trends as E', with the highest value found for dynamic control (6.68 ± 0.87 MPa).

After 14 days of culture, the results showed a slight increase in tan δ, dissipated energy and E" for cell-constructs, both static and dynamically cultured **(Fig 7**), compared to both static and dynamic controls. For E' and E*, the same trend was found after 14 days of culture, with

values for statically cultured cell-constructs of 4.24 ± 0.78 MPa and 0.44 ± 0.08 MPa respectively and 5.04 ±0.11 MPa and 0.49 ± 0.02MPa for cell-constructs under dynamic stimulation.

To better determine the effects of mechanical stimulation and cell culture on different groups, we decided to analyse the variation (V%) in the mechanical parameters (P) between the first cycle of day 2 (i.e. the first day of mechanical stimulation) and the last cycle of day 14 (i.e. the last day of culture) **(Fig 8)**.$V\% = 100*(P_{d14} - P_{d2})/ P_{d2}$.

Although not statistically significant, some trends are present in each of the mechanical properties analysed. In the absence of cells, all mechanical parameters presented lower percentages than those obtained in cell-constructs **(Figure 8)**. Tan(δ) presented a slight increase for static controls (4 ± 7%) and decreased for dynamic ones (-3 ± 6%). For dissipated energy, controls presented a decrease of -4 ± 17% and -10 ± 2% for static and dynamic conditions respectively. E'' presented the same trend as dissipated energy, with the highest decrease obtained for static conditions (-25 ± 4) compared to dynamic ones (-9 ± 6%). The elastic parameter (E') appeared to decrease particularly for the static control (-20 ± 9%), compared to a slight reduction for the dynamic control (-6 ± 0.1%). E* followed the same trends as E', with the highest decrease found with the static control with -20 ± 9% vs -6 ± 0.2% for the dynamic control.

In the presence of cells, Tan δ seemed to increase in both static and dynamic cultures, with an enhancement of 22 ± 16% and 16 ± 5% respectively. Dissipated energy presented a similar trend with enhancement of 25 ± 7% and 14 ± 9% for static and dynamic culture of cell-constructs, respectively. In terms of E'', cell-constructs presented an increase of 45 ± 5% for static and 31 ± 19% for dynamically-cultured cell-constructs. For E', dynamically-cultured cell-constructs presented the highest increase, with 16 ± 20%, while for static cultures a slight increase of 7 ± 6% was found. Regarding E*, the same trends appeared as for E', with the highest increase for dynamic conditions (16 ± 20%).

Static control appeared to have the lowest values for E', E" and E*, followed by dynamic control. On the other hand, both cell-constructs (dynamic or static cultured) presented the

highest enhancement of mechanical properties over time, with higher viscosity for static conditions and higher elasticity for dynamic ones

Discussion

Our scientific goal was to understand any correlation between the changes on the mechanical properties of the biohybrid scaffold and the activity of the seeded BMSCs, in association with the production and arrangement of the newly-formed ECM produced by cells. To the best of our knowledge, this analysis, which requested an adequate cell culture protocol, has not yet been investigated. Therefore, we first needed to confirm the role of mechanical stimulation on BMSC differentiation towards tendon lineage when cultured on adapted scaffolds.

In this study, we proposed to follow the cell-constructs' mechanical properties with a set of relevant parameters (E', E'' and tan δ) throughout the entire dynamic stimulation and key parameters of cell activity as proliferation, orientation differentiation. Our experiment, generating 5% of strain at 1Hz, represents a dynamic testing involving continuous repeated sine strain simulating physiological activities for tendons(20).

Our first concern was thus to develop a biomaterial suitable for this approach. Electrospinning has been widely used in the development of materials mimicking the fibrous nature of the tendon extracellular matrix(21). While our PCL scaffold presented an elastic modulus of 8.05 ± 0.82MPa **(Fig 3)**, which is still a long way from native tendon(22), the stress-strain curve revealed three regions similar to those found in tendons(4). Taking into account this stress-strain response, and comparing them with the mechanical behaviour of tendon, we decided to apply a cyclic sinusoidal strain of 5% (0-5%) corresponding to the heel region of both tendon and our electrospun scaffold. This was intended to calculate the viscoelastic behaviour of our cell-constructs when submitted to cyclic stretching with repeated stretching cycles and strain rates within a low range. The effect of this mechanical protocol was analysed for cell behaviour, mechanical changes over time and the correlation between both variables.

(1) Cell behaviour over mechanical stimulation

Regarding the cell behaviour, after one week of culture, there was no difference in terms of proliferation between static and dynamic conditions **(Fig 5#)**. After two weeks, the number of cells was found to have tripled for the static culture and even quadrupled for cells submitted to dynamic stretching. We can thus conclude that the mechanical stimulation enhanced the proliferative ability of BMSCs, inducing an increase in cell proliferation in response to mechanical load. This result is consistent with other experiments conducted with BMSCs, where more cells were found after 2 weeks of culture compared to shorter culture times under 5% of stretching(23). Other studies have shown similar results with fibroblasts(24) and TDSCs(25). While an increase in cell proliferation is found as a response to mechanical stimulation, the mechanisms involved in this mechano-response still need to be clarified(26).

When stimulated, BMSCs presented an elongated morphology aligned with the stretching direction **(Fig 5* B,D)**, while cells cultured under static conditions were randomly organised on the scaffold **(Fig 5* A,C)**. Similar behaviour was found in another study that analysed the impact of mechanical stimulation on cell alignment when cultured on randomly-oriented scaffolds in the same range of nanofiber size(27).

Regarding collagen synthesis, the cell-constructs showed continuous production over time, with more collagen found after 2 weeks of culture for each condition **(Fig 5§)**. After two weeks of culture, more collagen was found on cell-constructs subjected to mechanical stretching, in agreement with several other studies(28–30). This effect has been shown consistently in in vivo studies carried out on tendons where an increase in collagen synthesis was observed as a part of the tendon adaptation response to continuous mechanical loading(31).

Analysis of the ECM produced by cells in both static and dynamic cultures was also performed by immunofluorescence staining of type I collagen and tenomodulin **(Fig 5*)**. Collagen I is the most abundant collagen in tendons, and tenomodulin is a late tendon differentiation marker, and a key glycoprotein for the mature state of tendons(32,33). Both were more abundant under mechanical stimulation when compared to static culture samples. Furthermore, they appeared to be arranged in the stretching direction. Taken together, these data with cell alignment achieved in dynamic conditions, revealed a tendon-

like phenotype of BMSCs under dynamic culture conditions, similar to native tendon arrangement(34).

(2) Mechanical stimulation; impact of the culturing conditions.

Concerning the evolution of the biomechanical behaviour and to avoid the noise, we decided to analyse and represent the V% of the targeted parameters variations as described before with the equations, from the 1200 to 3600 sinus where a neat loop was observed, similar to the mechanical behaviour observed in tendons(35). For both static and dynamic controls, E', E'' and Tan δ seem to present a reduction while for cell constructs those parameters seem to increase over time **(Fig 8)**, which maybe result of cellular behaviour.

The elastic modulus E´ considers the elastic properties both from the material and for cells. The increase over time could be explained as the result of newly deposited type I collagen fibres. Type I collagen is effectively an important stress-bearing component of connective tissues. It is secreted by cells and hierarchically assembled into fibrils from a packaging of collagen molecules, embedded in a soft matter formed of water and proteoglycans. This organisation gives the collagen structure enough rigidity to be considered an elastic beam(36). In addition, the aligned orientation adopted by collagen fibres (Fig 5*) through the stretching direction could explain the higher E' percentage (16%) for dynamically-cultured cell-constructs compared to static culture (7%), as aligned collagen fibres are better suited up to supporting tensile stress(37).

The loss modulus (E'') takes into account the viscous behaviour, therefore its energy dissipation behaviour and confirmed our previous results. As seen before, when initial and last values were compared **(Fig 8)**, the controls without cells presented a reduction in E'' with -25% for static and -9% for dynamic controls.

The cell-constructs showed an increase of E" for both static and dynamic conditions. However, this increase seemed to be higher for static conditions with 45% increase, compared with 31% for dynamic cultures. Taken together, these results consistently demonstrated that cell-constructs, presented increased viscosity over time, compared to nude biomaterials. Finally, dynamic cultures exhibited a greater increase in the elastic properties of the cell-constructs, with a lesser gain in viscous properties, while the static

cultures had a greater influence on the viscous properties, with a lower increase in elasticity.

This effect may be explained by the effect of the alignment of the extracellular matrix, particularly the collagen fibres, which align through the tensile axis, resulting in an increase in the elastic properties of the cell-constructs exposed to dynamic culture conditions **(Fig 5*)**.

Others viscous related parameters as tan δ seem to be in accordance with these results. The value of tan δ is an indicator of how efficiently the material absorbs and dissipates energy due to fiber-cells rearrangements and internal friction. When we compared its evolution over time **(Fig 8)**, both controls presented the lowest values.

Cell-constructs, in both static and dynamic cultures, presented an enhanced tan δ of 22% and 16% respectively, compared to cell-free control scaffolds **(Fig 8)**. Because tan δ represents the ratio of Loss over storage modulus, the highest values of tan δ were related to a higher damping factor. In a rabbit Achilles tendon regeneration model, Nagasawa et al. found higher values of tan δ for regenerated tendons after surgery compared to controls(33). This increase was explained as the result of the neosynthesis of collagen fibres. As this "new" fibre presented a lower amount of mature cross-linking, collagen mobility was increased within the tissue, resulting in more energy dissipation. This may explain the increase in tan δ in our cell-constructs, where cells produced collagen, compared to controls without cells. In addition, the random organisation of collagen fibres for static conditions may explain the greater tan δ percentages. These results are in accordance with others mechanical parameters studied here (Supplementary data) as energy dissipation and E*.

Correlating both the biological and mechanical results (**Fig 9**), it seems that mechanical stimulation has a positive effect on cell proliferation and collagen synthesis, which was greater than static culture conditions. Cell and collagen alignment, together with an increase in tenomodulin under dynamic culture conditions, provides a better environment for BMSCs to differentiate towards a tendon-like phenotype. Furthermore, the mechanical properties of the cell-constructs were enhanced over time compared to the control scaffolds without cells. This could be explained as a result of the cellular activity, translated into the production of an ECM. By synthesising collagen, which in tendons and ligaments is responsible for its mechanical properties, the viscous properties (tan delta, dissipated

energy and E'') were increased in both the static and dynamic cultures. The random nature of this ECM conferred higher viscous properties for cells cultured under static conditions, explained as an increase in the mobility of collagen and the associated water molecules within these random fibres, while dynamically-stimulated cell-constructs presented aligned collagen fibres towards the direction of the stretching. These aligned fibres could better retain water molecules, resulting in less mobility compared to random ones. In addition, due to the elastic nature of the collagen fibres when they are stretched, the alignment may explain a greater increase in elasticity (E') under mechanical cultivation.

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Data availability

The data supporting the findings of this study are available from the authors upon reasonable request.

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Author contributions

A.G.G conceived, designed and performed the experiments. J.B.P developed the mechanical data analysis and assisted with in vitro mechanical stimulation. M.B.L was involved with cell culture maintain. S.L.R and M.N provided support and information regarding cell extraction. Q.D provided technical support with Bose BioDynamic 5100. C.L and F.B provides scientific directions and analysis of the data. A.G.G, C.L and F.B wrote the manuscript.

Competing interests

The authors declare no competing interests.

Figure legends

Figure 1. Electrospinning device.

Figure2. Experimental plan design. Correlation between cellular activity (Cell Scale T6) and biomechanical performance (Bose Biodynamic 5100).

Figure3. SEM pictograph of a 10-wt % PCL scaffold **(A)**. Fibre orientation distribution in PCL scaffolds **(B)**.

Figure 4. Stress (MPa) vs strain (%) curve for 10-wt % PCL scaffolds in a dry (dark grey) or wet (light grey) state, obtained using the Electroforce 3200 system.

Figure 5. Cell proliferation (#) and hydroxyproline synthesis (§) over time, at 1 and 2 weeks of

static and dynamic culture performed with T6 CellScale. Hydroxyproline concentration was related to collagen content (*** for $p<0.001$). (*) Fluorescence staining of the actin cytoskeleton (red) (A-D), type-1 collagen (green) (E-F) and tendomodulin (I-L) for static or dynamic culture performed for 1 or 2 weeks). Cell nuclei were stained blue as a counter-stain.

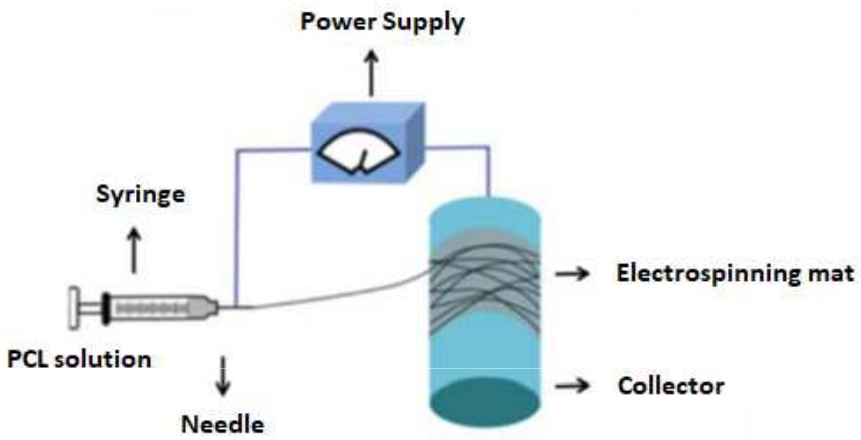
Scale bar of 50µm.

Figure 6. Design of the dynamic culture process during 12 days of stimulation **(A)**. Representative stress vs strain curve **(B)** of the first sinus from the first cycle and the last sinus from the last cycle for cell-constructs subjected to dynamic culture conditions.

Figure 7. Tan δ, Energy dissipation values, E*, E' and E'' for both dynamic and static conditions cultured with or without cells for both days 2 and 14. Tests were performed in the Bose BioDynamic 5100. Values represent the arithmetic average for each value from 1200 to 3600 sinuses (n=3). There was no statistical difference between the conditions.

Figure 8. Evolution of the Tan δ **(A),** dissipated energy **(B),** E´**(C)** and E´´ **(D)** over time expressed in terms of variation (V%). V%= ((Last cycle – First cycle) / (First cycle))*100.

Figure 9. Schematic representation of evolution in the mechanical properties for cellular constructions in the absence or presence of mechanical stimulation.



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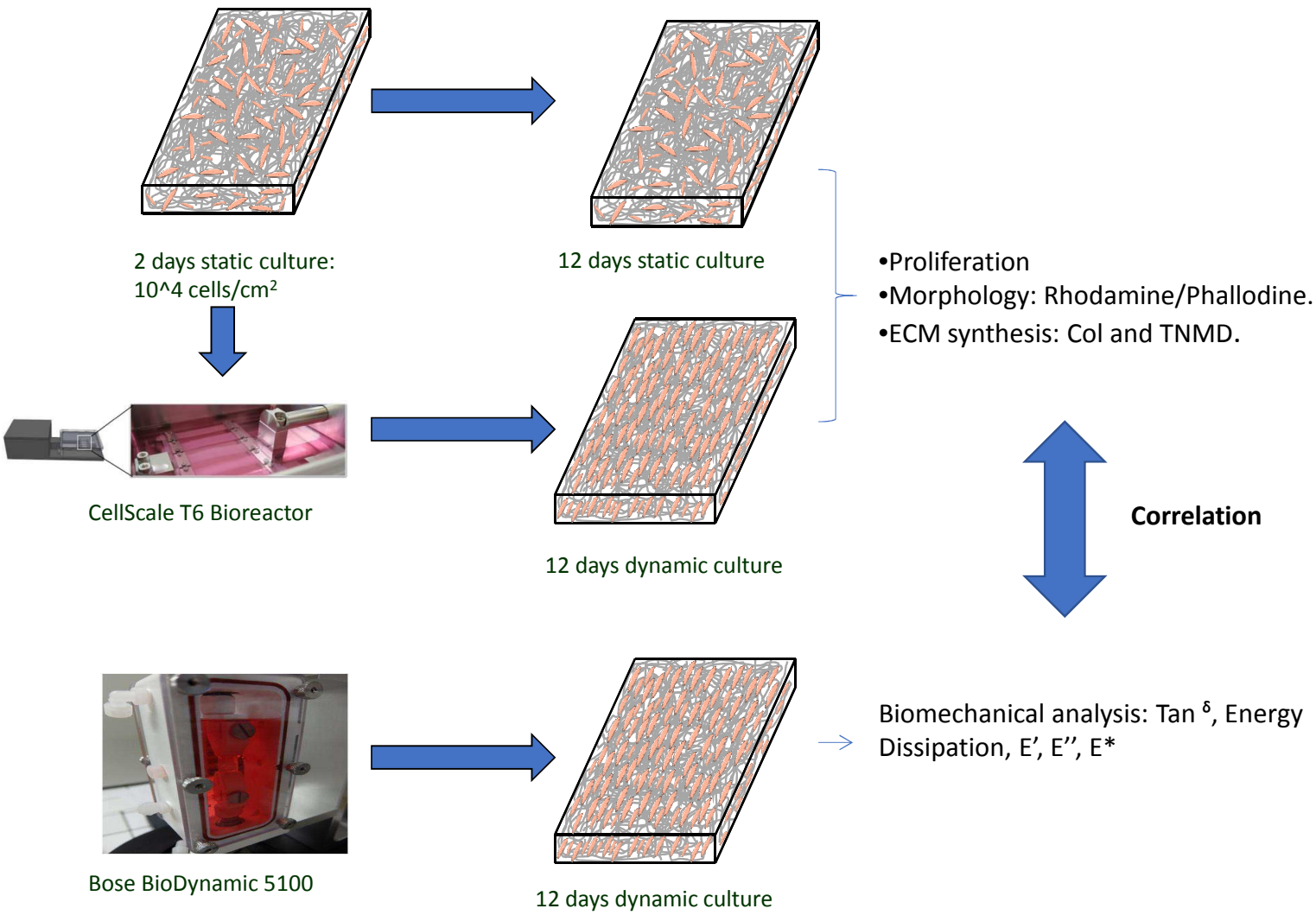
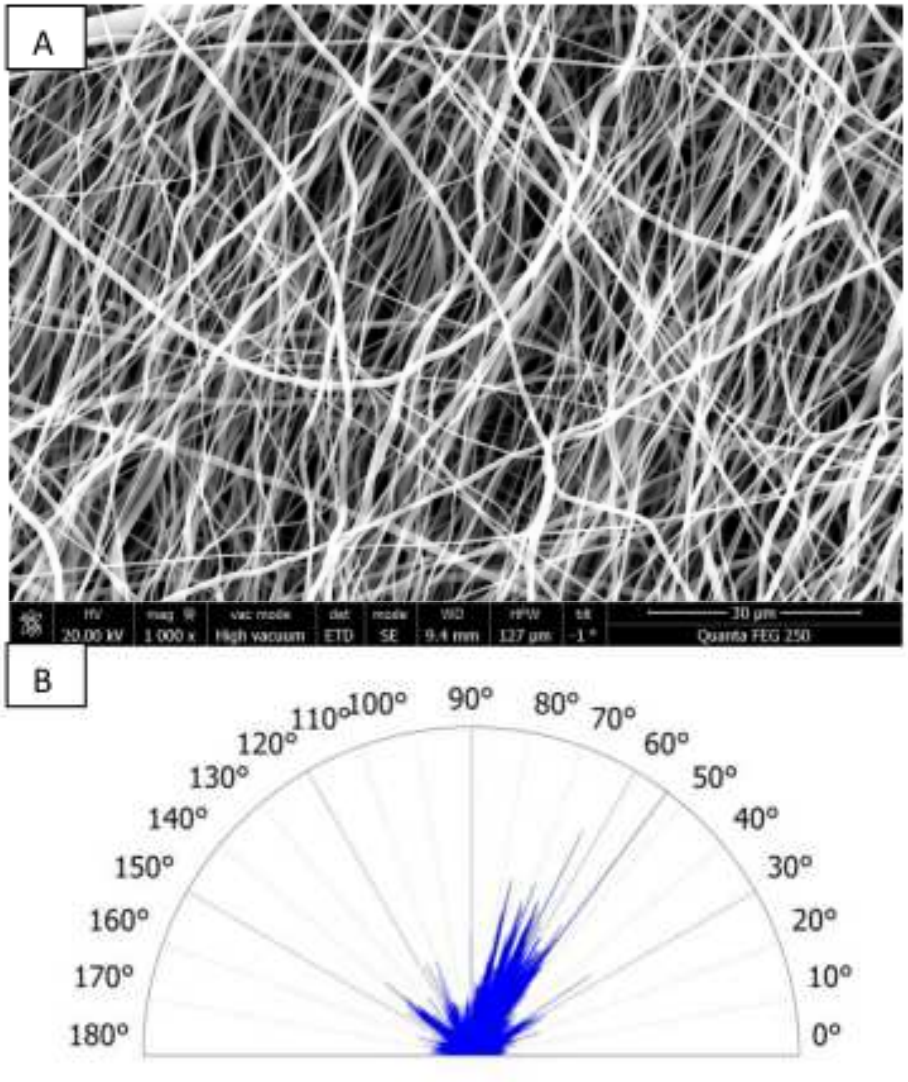


Figure 2. Experimental plan design. Correlation between cellular activity (Cell Scale T6) and biomechanical performance (Bose Biodynamic 5100). Briefly, after 2 days of static culture, scaffolds were transferred to the different bioreactors (Electro Force and Bose Biodynamic). Once attached, the dynamic culture was launched for 2 weeks.



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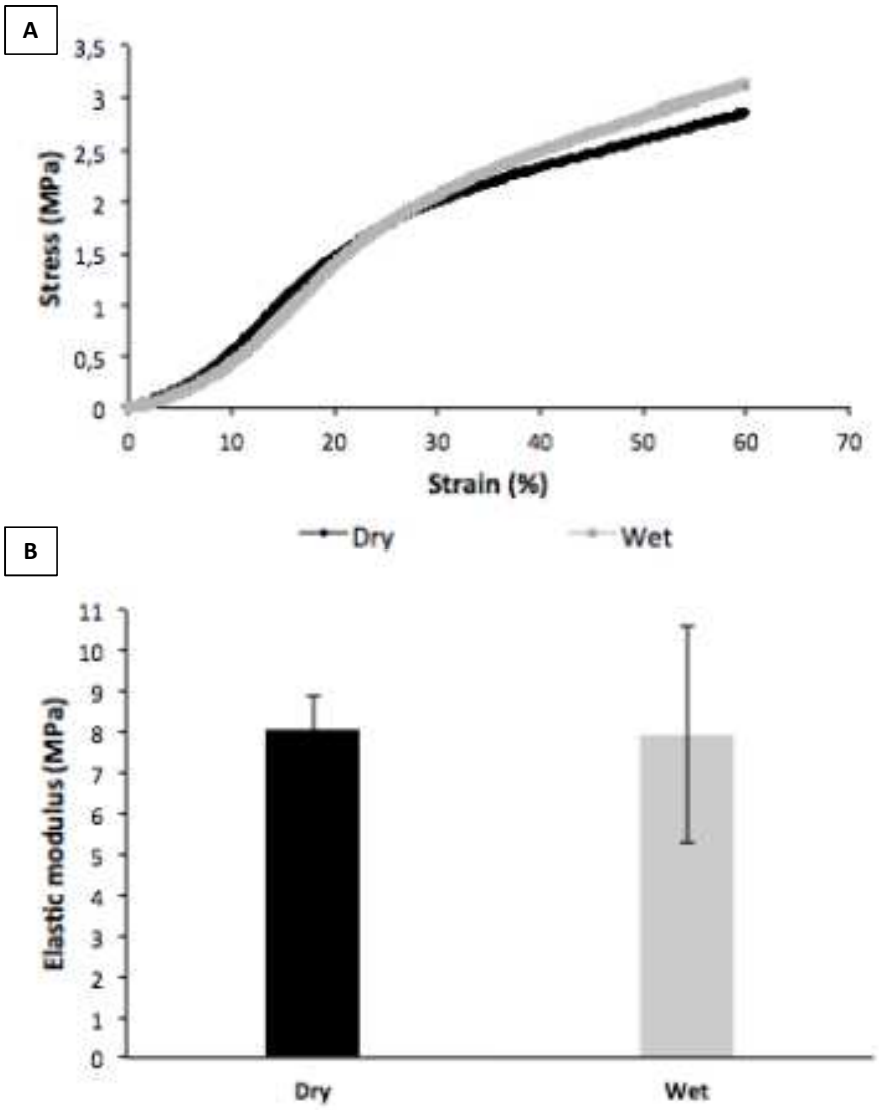

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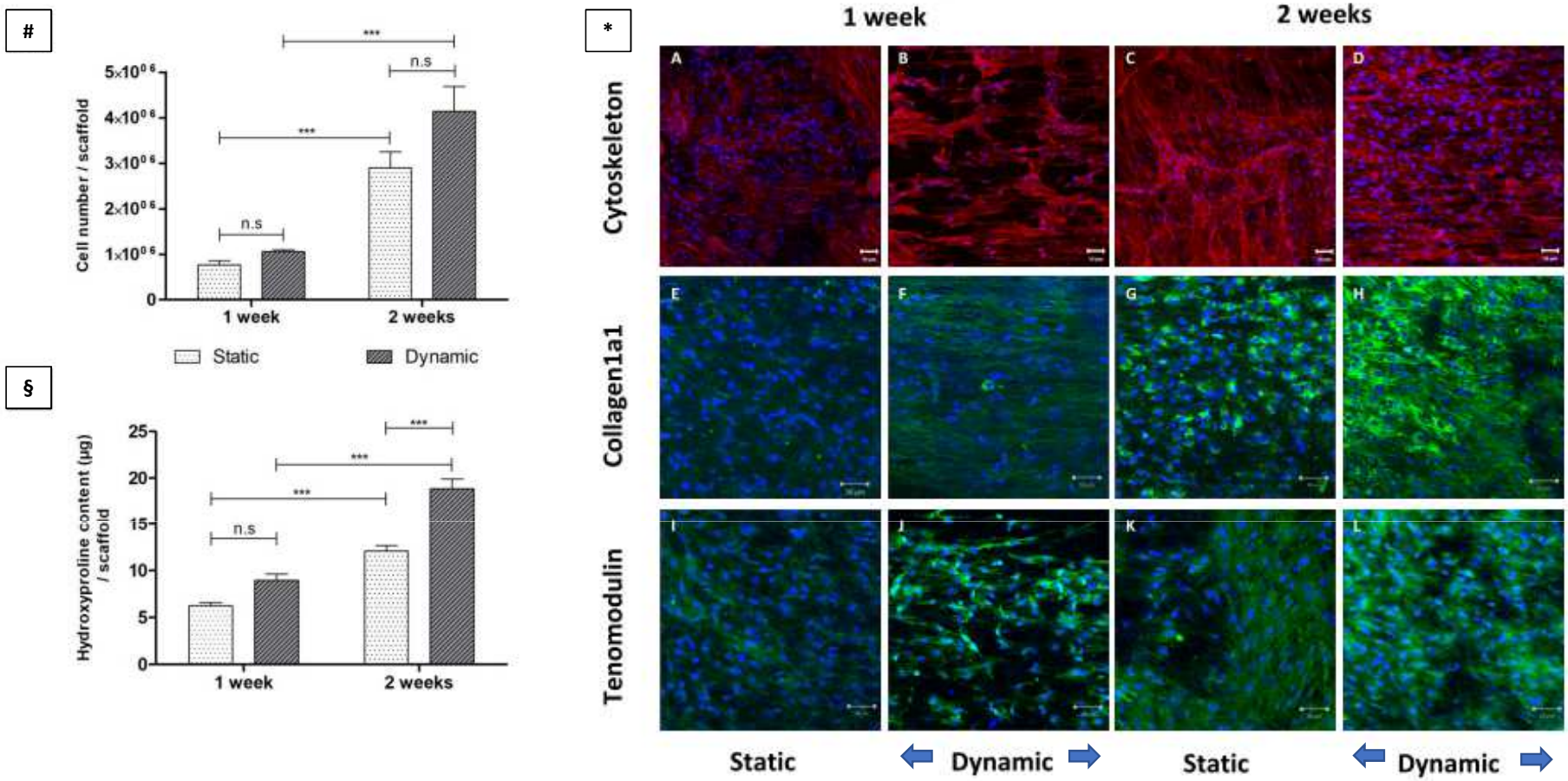

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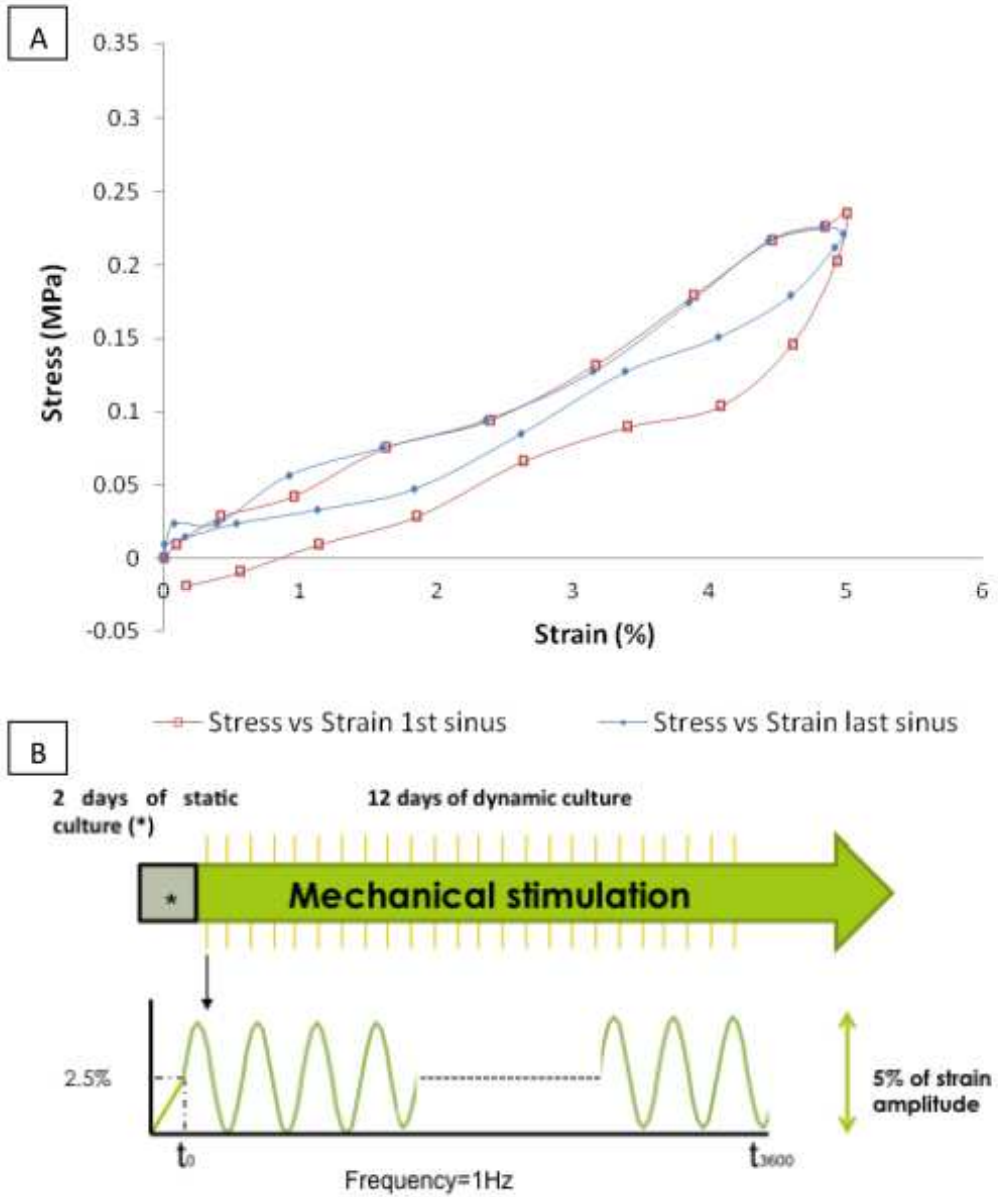
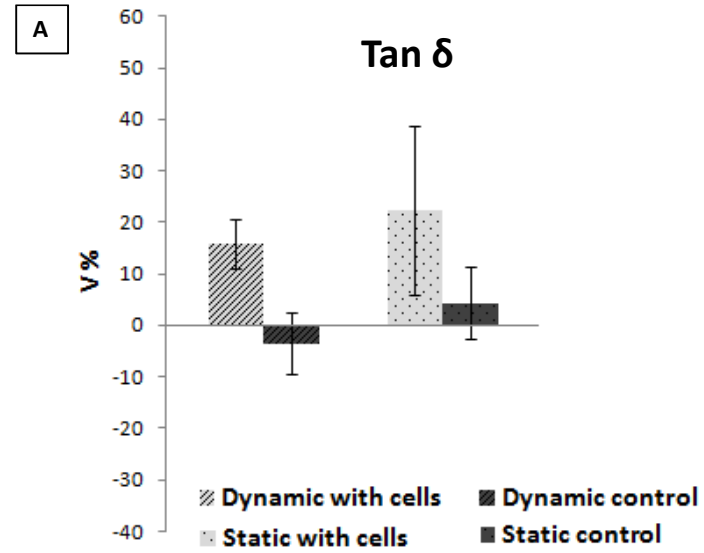
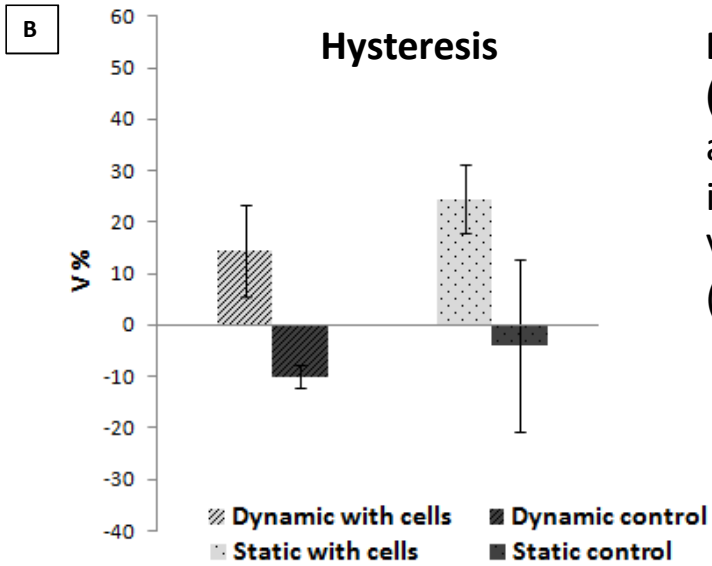


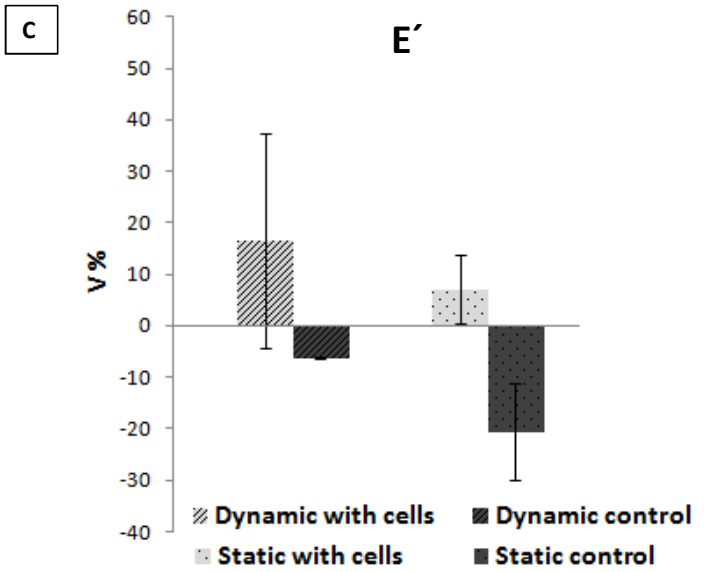
Figure 6. Design of the dynamic culture process during 12 days of stimulation **(A)**. Representative stress vs strain curve **(B)** of the first sinus from the first cycle and the last sinus from the last cycle for cell-constructs subjected to dynamic culture conditions.
After 2 days of static culture, the cell-construct or the control scaffolds were fixed inside the bioreactor chamber and the mechanical test was launched. Both signal force and displacement were recorded over time and found to be smooth, without any background noise, making it possible to monitor mechanical (elastic and viscoelastic) properties.

	Tan delta		Hysteresis		E* (MPa)		E' (MPa)		E'' (MPa)	
	2 days	14 days	2 days	14 days	2 days	14 days	2 days	14 days	2 days	14 days
Static with cells	0.08± 0.01	0.10± 0.01	0.64± 0.35	0.80± 0.14	3.97± 2.27	4.27± 0.79	3.95± 2.26	4.24± 0.78	0.30± 0.16	0.44± 0.08
Static control	0.12± 0.03	0.12± 0.02	0.92± 0.14	0.81± 0.11	4.03± 0.92	3.46± 0.43	3.90± 0.91	3.42± 0.43	0.42± 0.07	0.38± 0.06
Dynamic with cells	0.09± 0.01	0.10± 0.06	0.90± 0.11	1.02± 0.12	4.34± 1.21	5.07± 0.11	4.32± 1.20	5.04± 0.11	0.37± 0.13	0.49± 0.02
Dynamic control	0.09± 0.03	0.09± 0.03	1.47± 0.57	1.32± 0.48	6.68± 0.87	6.24± 0.83	6.63± 0.89	6.21± 0.84	0.59± 0.14	0.53± 0.10

Figure 7. Tan δ, Energy dissipation values, E*, E' and E'' for both dynamic and static conditions cultured with or without cells for both days 2 and 14. Tests were performed in the Bose BioDynamic 5100. Values represent the arithmetic average for each value from 1200 to 3600 sinuses (n=3). There was no statistical difference between the conditions.







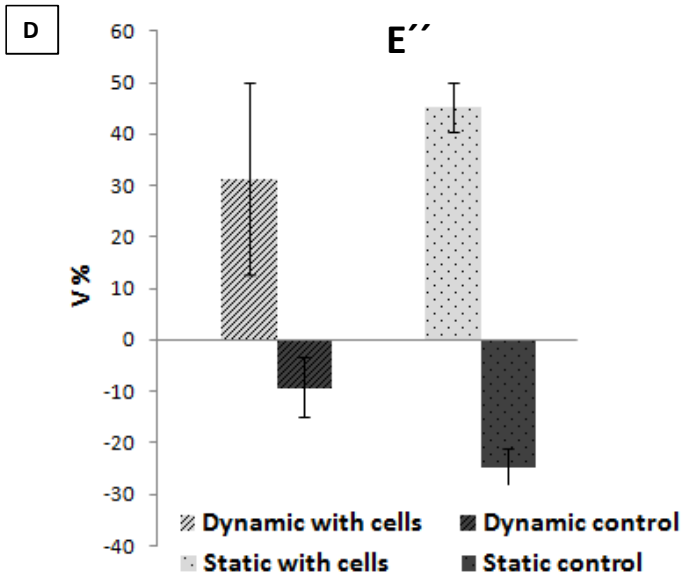
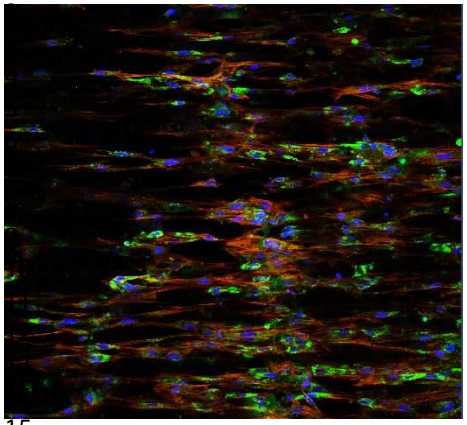
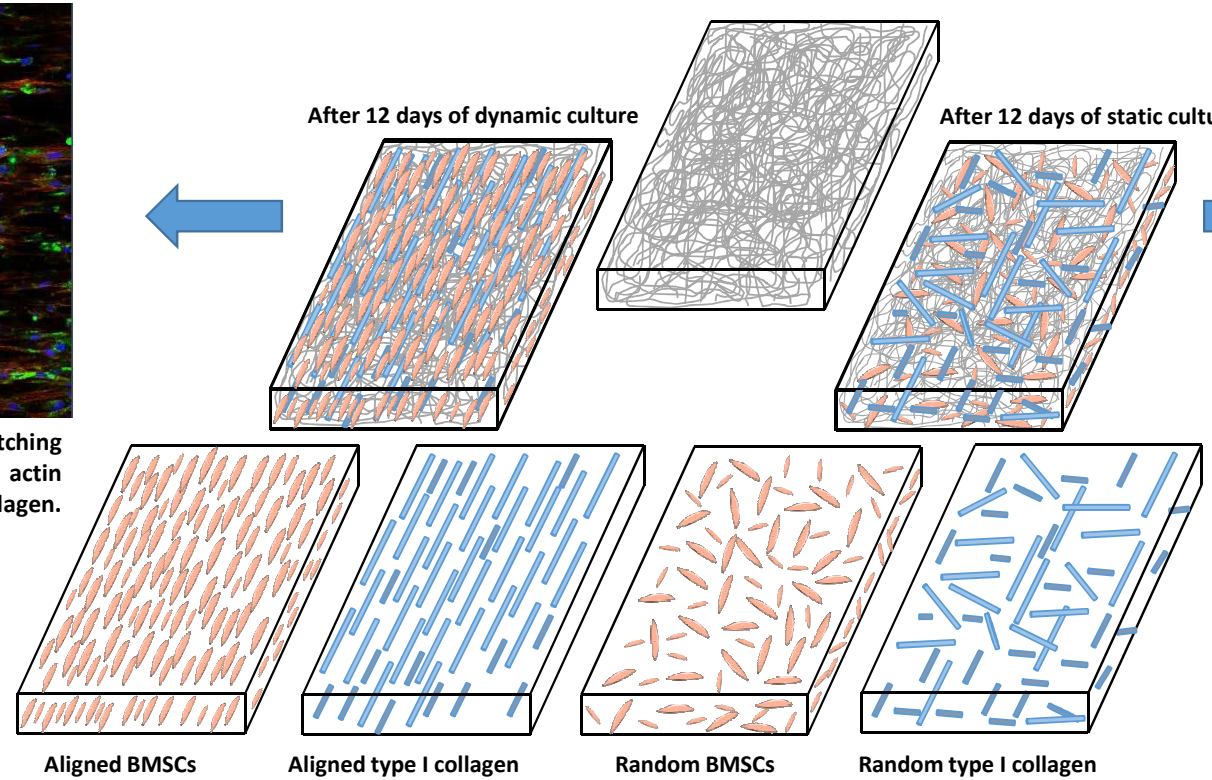
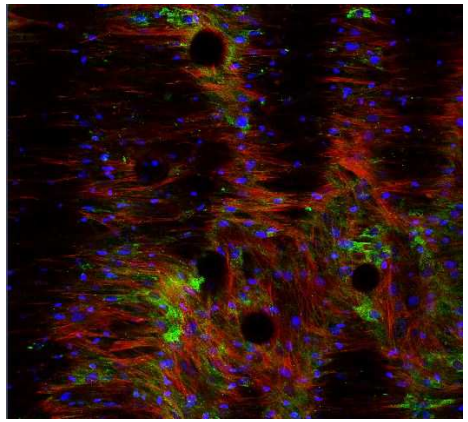


Figure 8. Evolution of the Tan δ **(A)**, dissipated energy **(B)**, E´**(C)** and E´´ **(D)** over time expressed in terms of variation (V%). V%=((Last cycle – First cycle) / (First cycle))*100



Aligned cells along the stretching direction. Blue: cells nuclei; red: actin cytoskeleton; green: type I collagen. Single stack image.





Randomly organized cells. Blue: cells nuclei; red: actin cytoskeleton; green: type I collagen. Single stack image.

	Tan delta	E´	E´´
Static with cells	↑↑	↑	↑↑
Dynamic with cells	↑	↑↑	↑

Figure 9. Schematic representation of evolution in the mechanical properties for cellular constructions in the absence or presence of mechanical stimulation.