Araque Monrós, MC.; Gil-Santos, L.; Monleón Pradas, M.; Más Estellés, J. (2020). New bioreactor for mechanical stimulation of cultured tendon-like constructs: design and validation. Expert Review of Medical Devices. 17(10):1115-1121. https://doi.org/10.1080/17434440.2020.1825072

New bioreactor for mechanical stimulation of cultured tendon-like constructs. Design and validation.

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Abstract

Objective: Although several different types of bioreactor are currently available with mechanical stimulation of constructs or prostheses for tendon regeneration, they are in many cases expensive and difficult to operate. This paper proposes a simple bioreactor to mechanically stimulate up to three constructs for tendon and ligament repair, composed of a stainless-steel frame and an electric motor.

Methods: The deformation is produced by a cam wheel, whose eccentricity defines the maximum deformation. The test samples, braids of PLA seeded in surface with mouse fibroblasts, are immersed in the culture medium during mechanical stimulation.

Results: Its advantages over existing similar bioreactor designs include: easy renewal of the culture medium and an external electric motor to avoid heating and contamination issues.

After 14 days of stretching, the culture samples showed enhanced cellular proliferation and cell fibre alignment in addition to higher production of type I collagen. The cells initially seeded on the braid surface migrated to the inside of the braid.

Conclusion: Although the results obtained have a poor statistical basis, they do suggest that the bioreactor could be usefully applied to stimulate constructs for tendon and ligament repair. Anyway, further experiments should be conducted in future.

Keywords: bioreactor**;** mechanical stimulation; construct; regeneration; tendon.

1. Introduction

Prostheses have been used to restore damaged tendons and ligaments over the past century. These tissues can be replaced either by natural fibres (silk, catgut or grafts (autografts, allografts or xenografts) and synthetic materials (nylon, polyethylene, silastic, Teflon, PLA, PGA, etc.) [1-4], although this solution presents certain long-term problems, like loosening of the bone anchorage, slackness of the prosthesis, or stiffness of the member [5, 6]. Regenerating the native tissue with implants focuses on Tissue Engineering techniques, based on a combination of cellular cultures and biomaterials [7-11].

The mechanical conditions under which this regeneration process occurs are especially important. It has been repeatedly shown that regeneration of this tissue is more effective when the cells are mechanically stimulated, as in the body's natural reaction to an injury. Cell growth on non-stressed supports leads to deficiently organized tissues, while the application of a mechanical force during regeneration improves the properties of the regenerated tissue [12-18], mainly due to the collagen fibres of stressed tissues being aligned in the direction of the force applied and the increased production of type I collagen.

In vitro experiments should resemble as much as possible the *in vivo* conditions of the biological processes during regeneration (acidity, the medium surrounding the tissue, nearby tissues, mechanical stress, anchorage to muscle and bone, etc.). Bioreactors aim to achieve similar ambient conditions for the cells. Cell cultures should be carried out in controlled conditions in a sterile environment, as regards temperature, load ranges and deformation, etc. [19]. Bioreactors should also be able to deal with several samples at once.

During mechanical stimulation, stresses are transferred to the cells through the biomaterial support on which they are seeded. Different types of bioreactors can be used for different types of tissues [18, 20-29]. The aim of this work was to design, build and validate an easily operated bioreactor with an external motor that could be used for tissue engineering techniques to mechanically stimulate cell-biomaterial constructs for tendon and ligament regeneration. This *ex vivo* partial tendon and ligament tissue regeneration would be an advantage during subsequent surgical implantations, leading to better integration of the prostheses in the host body.

2. Materials and methods

The bioreactor is made up of a stainless steel frame, capable of being sterilized before each experiment, either in an autoclave or sanitized with ethanol solution. One end of the sample is anchored to the fixed part of the frame and the other to the moving part, which is oscillated by an adjustable cam wheel (its eccentricity defines the maximum deformation) to subject the sample to variable stretching cycles. To avoid the contaminating the cells under culture, the motor is outside the incubator and transmits the movement by a steel cable inside a sheath. The culture medium is placed in a Petri dish, so that the samples subjected to stretching cycles remain immersed in the culture medium, which can be renewed as frequently as required by simply replacing the Petri dish.

The motor can be placed as far from the incubator as needed to avoid heat transfer or contamination. The appropriate temperature and ambient conditions can be set up inside the incubator.

2.1 Bioreactor. Frame design.

The 180x85x55 mm^3, 304 medical grade stainless steel frame was supplied by Mecanizados, S.A. (Alfafar, Valencia, Spain). The glass tubes were manufactured by VidraFoc (Valencia, Spain). Figure 1 shows a side view, plan and photo of the bioreactor.

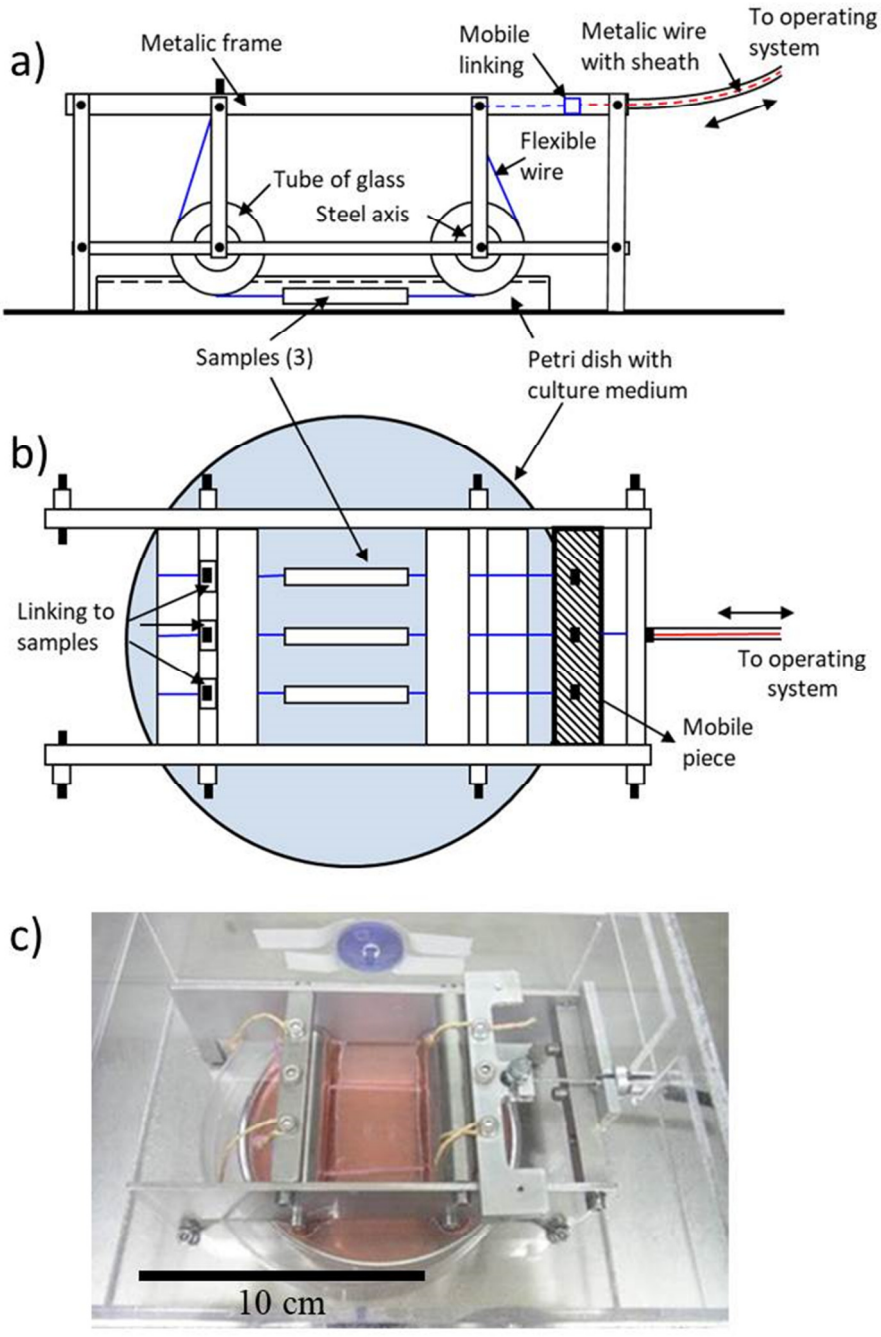

Fig. 1 a) Bioreactor side view, b) plan and c) photo. The two samples at the left end are oscillated by the moving part on the right. Petri dish containing the culture medium can also be seen.

The samples are tied to two cables with very high rigidity anchored to the frame in such a way that practically all the deformation is absorbed by the sample. The moving part of the frame is oscillated by an external motor and applies tensile forces to the samples. The frequency of stretching cycles can be controlled by adjusting the motor speed. To avoid metal parts coming in contact with the culture medium, the steel cables slide over two glass tubes. Two steel rods (10 mm outer diameter) pass through the glass tubes (20 mm outer and 10 mm inner diameter) to support the forces on the samples and avoid breaking the glass.

The samples can be of any length, although only the part immersed in the culture medium can be seeded with cells. The samples remain immersed in the Petri dish containing the culture medium. Three samples can be tested simultaneously.

A methacrylate box (220x180x80 mm^3) was designed to minimize the risk of sample contamination and facilitate the transport of the bioreactor from the laminar flow chamber (where the samples are attached to the frame) to the incubator chamber.

2.2 Bioreactor. Power system.

The samples are tensed at one end by a unit containing a 12 V (maximum voltage) DC electric motor and a gearbox (gear ratio r=104) Beru A350 (BorgWarner Inc. Auburn Hills, (WHQ) MI, USA), as shown in Figure 2.

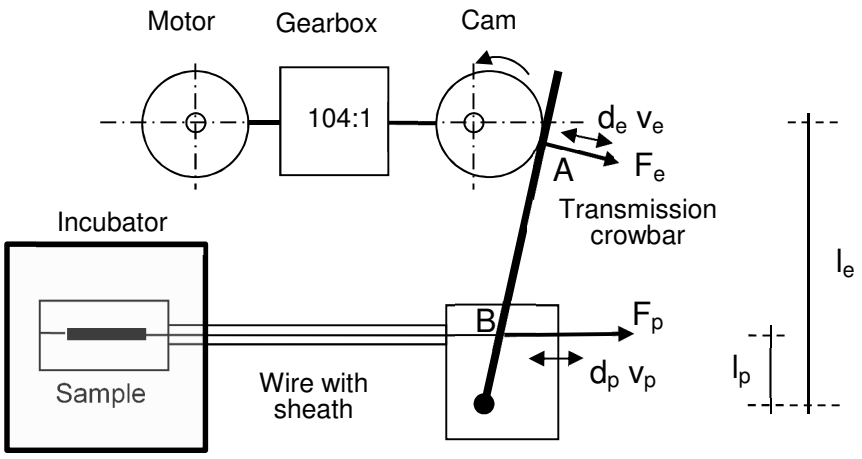

Fig. 2 Bioreactor power system

The cam wheel is turned by a shaft from the gearbox, that in turn moved a bar (contact point A). Such bar, at point B, moved the wire connecting the mobile piece of frame of bioreactor. Therefore, depending on the amplitude of movement of cam (d_e), on the lengths l_e and l_p, on the intensity consumed by the motor (I), on the necessary intensity to overcome every mechanical friction (I_0), and on constant of motor k' (relationship between torque and current, k'=0.0155 T·m^2), we can obtain the force applied to stretch the sample (F_p) and the amplitude of the applied deformation (d_p):

$$F_p = \frac{r \cdot k'(I - I_0)\pi}{d_e} \frac{l_e}{l_p} \qquad (1) \qquad\qquad d_p = \frac{l_p}{l_e} d_e \qquad (2)$$

The motor is powered by an adjustable DC Gold Source Power Supply DF1731 SB 0-30 V, 0-3 A Dual Out (Apliquem Micrones 21, Valencia, Spain). The system only works in the displacement-control mode. The maximum sample displacement can be

varied by installing a cam wheel with a different eccentricity. The frequency of the movement applied to the sample can be controlled by altering the applied voltage.

2.3 Bioreactor calibration

The initial deformation applied to the sample was lower than expected due to its sheath being too elastic. It was therefore necessary to calibrate the bioreactor by mounting a load cell U3 (HBM Ibérica, Madrid, Spain) with a range 0-200 N and a resolution of 0.1 N, and a displacement sensor (a ruler with a resolution of 0.25 mm) to measure the magnitudes really applied to the samples. These sensors were then disassembled and no real time acquisition was possible.

When the power supply was adjusted to 6 V, the samples were subjected to a stretching movement of frequency 0.3 Hz. The maximum load applied to the samples was 10 N and at an amplitude of 1 mm, or 2% strain, according to the length of the tested sample (50 mm). The strength and deformation values were between the usual values quoted in the literature [15, 17]. As the motor can supply a maximum 12 V and the revolutions are directly related to the applied voltage, the frequency of the movement can reach up to 0.6 Hz. The width of movement only depends on the cam wheel eccentricity, while the force applied to the sample depends on the displacement and on the features of the sample. This force applied to the sample could vary during the test due to stress relaxation because of the polymeric nature of the sample or even to the extracellular matrix maturation process, but the strain will remain unchanged in both cases.

2.4 Static and dynamic cell cultures

Dynamic tests were carried out to verify the bioreactor's ability to mechanically stimulate the seeded samples and the effective transfer of the mechanical stimulus to the

cells. Also static tests were carried out. Other aims were to verify that the bioreactor could be easily operated when the culture medium was changed and that the external motor avoided contamination and temperature rises. The samples tested were made up of hollow PLA braids seeded on the surface with mouse fibroblasts (cellular line L929 in passage 3). These braids have been self-manufactured and have been proposed as elements of a construct intended for tendon regeneration. The materials, manufacturing process and physicochemical properties can be found in [8, 30]. Culture times were 7 (T7) and 14 (T14) days.

The 100 mm long PLA braid was tied at both ends to a 50 mm long Kevlar braid. The PLA sample was sanitized with EtOH 70% solution, conditioned overnight with PBS and DMEM with 4.5 $g \cdot l^{-1}$ D-glucose without FBS for 24 h before cell culture. The samples were then surface-seeded with 100 µl of a cellular suspension ($1x10^5$ cells).

The samples were incubated at 37°C and 5% CO_2 foralong 1 h, mounted in the previously sterilized bioreactor, and covered with culture medium (approx. 110 ml) (n=2). The set was carried to the incubator and connected to the motor. This connection is easily made by inserting the head of the wire into a hole on the moving part of the frame. The culture medium was renewed every three days by replacing the Petri dish under the frame of the bioreactor, and mechanical stimulus was applied 9 h per day, with frequency of 0.3 Hz, maximum load 10 N, and 2 % maximum strain.

The braided material without mechanical stimulation was used as the control sample (static test) in a Petri dish in the same conditions as the dynamic tests (same cell type and equal time points).

2.5 Analyses of cultured samples

The static and dynamically cultured samples were then subjected to standard morphology and immunofluorescence tests [30].

2.5.1 Morphology

Scanning electronic microscopy (JEOL JSM-5410, Japan) was used to observe the surface morphology of the braided PLLA. The samples were washed twice in phosphate buffer solution (PBS) and fixed with a solution of glutaraldehyde 2.5 % for 1 h at 4ºC, then washed 3-4 times with PBS at 5 min intervals. Prior to SEM observation, the samples were dehydrated by changing the water for EtOH. The samples were dried and gold sputtered for further analysis. Micrographs were taken at 10 kV.

The outer braid surface was studied for static and dynamic cell cultures at T7 and T14. To observe the internal braid morphology, the sample was cut lengthwise with a scalpel. The inner braid surface of the dynamic cultures was observed at T14.

2.5.2 Immunofluorescence

Cell distribution, morphology and type I collagen were examined by immunofluorescence analysis using a confocal microscope with inverted laser (Leica TCS SP2 AOBS, Germany).

The samples were washed with phosphate buffer solution (PB 0.1 M) for 5 minutes at room temperature. Blocking buffer was added and maintained for 2 h, after which the primary and secondary antibodies (Col I:PB ratio 1:40 and Cy3:PB ratio 1:200) were successively added (10 µl per sample of bodipy-FL phalloidin (Invitrogen) was also added to stain the cytoskeleton), incubated in the dark at room temperature and washed three times with PB 0.1 M every 5 minutes at room temperature. The nuclei were then stained with a solution of DAPI-Vectashield (Vector Laboratories) 1:1000 (DAPI:H_2O)

and incubated for 15-30 min in the dark. The solution was then removed and the samples washed three times every 5 minutes with PB 0.1 M and mounted on microscope slides with a drop of medium.

Static and dynamic cell cultures were observed at T7.

3. Results and discussion

After 7 and 14 days culture, the samples were fixed and the morphology of fibroblasts seeded on the braid surface was analysed by SEM. The main objective was not to analyse the changes in the samples, but to verify the bioreactor's performance and the effects of the mechanical stimulation.

The 7 and 14-day static and dynamic cell culture micrographs can be seen in Figure 3. The dynamic culture cell density is clearly higher than that of the static cell cultures for the same time (see figures bI, bII and bIII versus figures aI and aII for T7, and figures bIV, bV and bVI versus figures aIII and aIV for T14). The dynamically cultured cells can be seen to adhere to the entire braid surface. Increased cell density and better cell alignment in the direction of the fibres can also be seen with a more extended and fusiform morphology in the dynamic cultures.

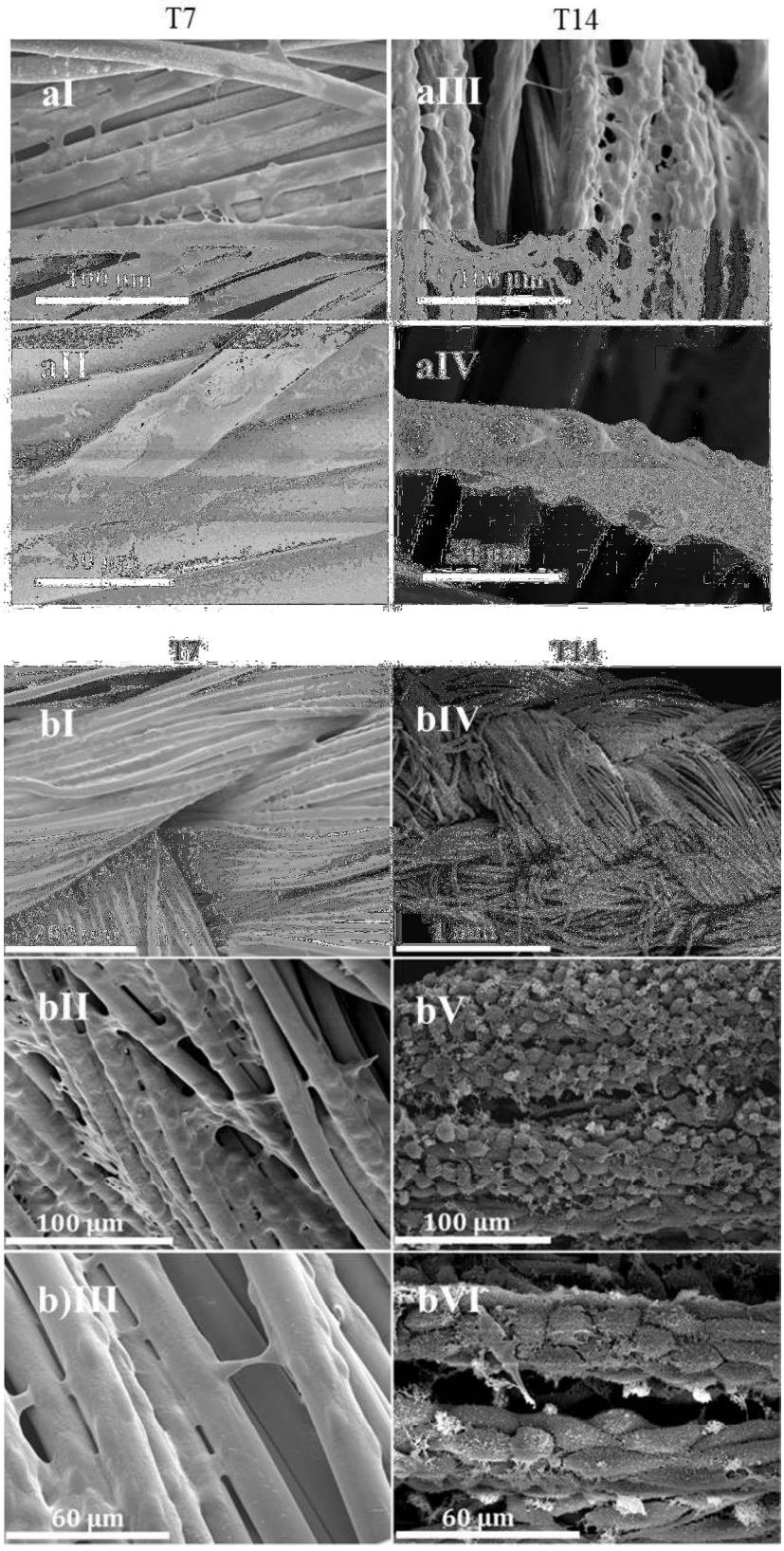

Fig. 3 SEM images of morphology of L929 cells on PLA braid surface after 7 and 14 days (T7 and T14) of static culture (set a) and dynamic culture (set b), with different

magnifications: very low (bI and bIV), low (aI, aIII, bII and bV) and high (aII, aIV, bIII and bVI).

The inner part of the dynamically cultured braid was also analysed by SEM images (Figure 4 a and b), which revealed the presence of internal cells, thus suggesting that the cells had migrated from the surface. As happened outside the braid, the cells exhibit an extended morphology in the same direction as the microfibres.

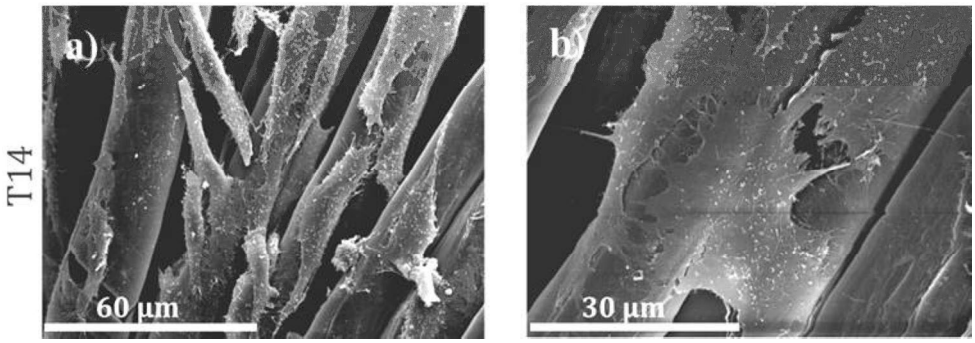

Fig. 4 SEM images of L929 fibroblasts morphology inside the PLA braid after 14 days of dynamic cell culture with two different magnifications: a) low and b) high.

Figure 5 shows immunofluorescence images after dyeing the cytoskeleton with actin and the nuclei with DAPI. The cells subjected to a mechanical stimulus seem to express more type I collagen than those cultured in static conditions (see figures bI, bII and bIII versus figures aI, aII and aIII), as reported by other authors [31, 32].

Type I collagen is the predominant protein of tendinous tissue and its expression and secretion are key factors in the regeneration process of this tissue [33, 34]. In both cases, a well-developed actin cytoskeleton can be seen.

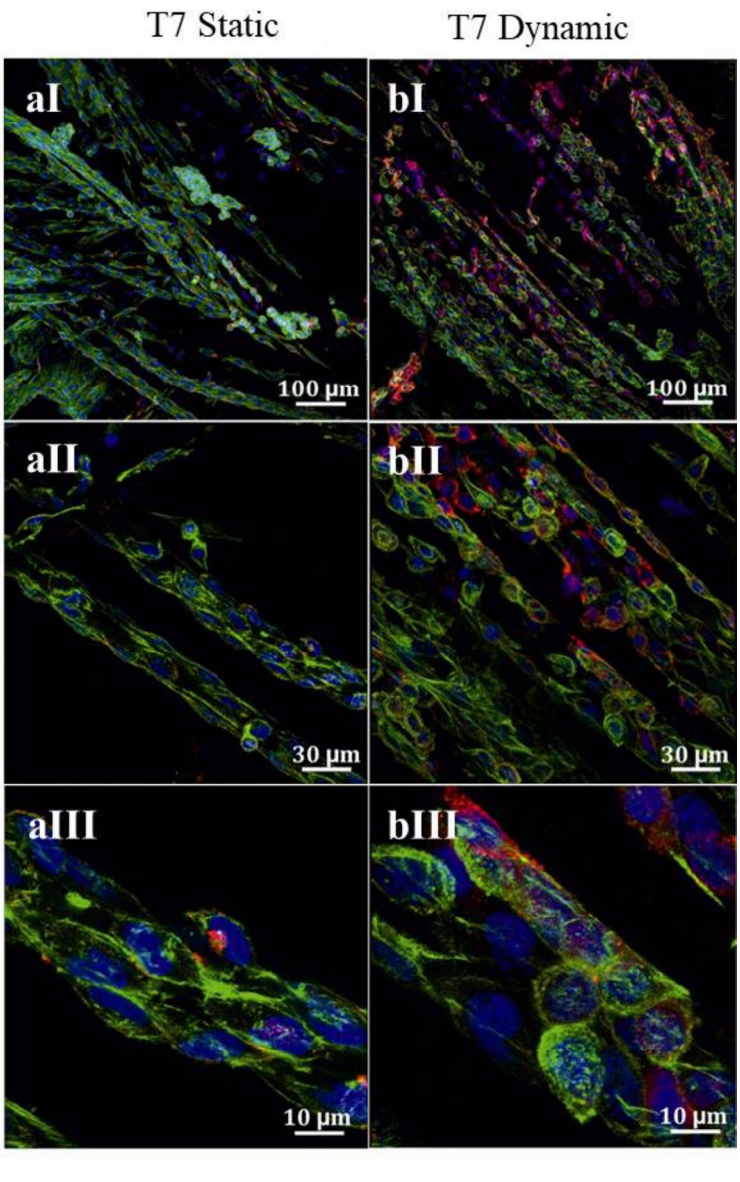

Fig. 5 Immunofluorescence images of type I collagen (red) and dyed actin cytoskeleton (green) of fibroblasts seeded on the PLA braid after 7 days of static (set a) and dynamic (set b) cultures with different magnifications: low (aI and bI), high (aII and bII) and very high (aIII and bIII). The cell nuclei were dyed with DAPI (blue).

Even though these results are only preliminary results, they show that the prototype bioreactor mechanically stimulated the tendon and ligament constructs, produced greater cellular proliferation, cell alignment in the direction of the fibres and cell migration to the inside of the sample.

The cell alignment indicated that the stress applied to the fibres was transmitted to the cells and increased the quantity of type I collagen.

4. Conclusions

Even though our tests were not statistically significant because of their insufficient number, they do suggest that the proposed bioreactor mechanically stimulated the tendon and ligament constructs. The strain applied to the sample can be easily modified by simply changing the cam wheel. The bioreactor also has the important advantages over those currently available of being easy to operate and its easy renovation of the culture medium. Further experiments should be conducted in order to state the effectiveness of the bioreactor.

Acknowledgments

This work was carried out thanks to the financial support of AITEX (Alcoi, Alicante, Spain), through the research contract "Development of braided biomaterials for biomedical applications".

The authors thank John Wiley and Sons for the permission to reprint part of Figure 5.

Conflicts of interest: None

Funding: Researching contract "Development of braided materials for biomedical applications".

Ethical approval: Not required

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