Bioreactors as physiological-like *in vitro* models

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Abstract

Bioreactors are powerful tools for *in vitro* development of engineered substitutes through controlled biological, physical, and mechanical culture conditions: bioreactor technology allows a closer *in vitro* replication of native tissues. One of bioreactors applications is the design of *in vitro* 3D tissue models as a bridge between 2D and *in vivo* models, allowing the application of 3R (replacement, reduction, refinement) principle. To this aim, bioreactors can be used to culture cells seeded on engineered scaffolds under *in vivo*-like conditions. Another key use of bioreactors is for perfusion decellularization of tissues and organs to be used as scaffolds. This contribution describes a *dynamic stretching.* bioreactor, imposing a mechanical stretching to the cultured constructs, allowing the development of skeletal muscle engineered constructs, and a *decellularization* bioreactor, designed for decellularization of blood vessels.

Introduction

Bioreactors, being very useful tools for developing and maintaining cell cultures on 3D scaffolds, represent highly functional platforms for the controlled imparting of microenvironment stimulation.[1] These systems can be extremely helpful also to reduce the recourse to animal studies, which are biologically accurate but extremely expensive, imply strong ethical issues and provide limited control on the experimental conditions due to the high inter-subject variability and to the inability of real time observations. This equipment facilitates the development of new and safe medical technologies to identify innovative pharmacological treatments that would allow the prevention or mitigation of harmful effects associated with different pathologies. In addition, bioreactors find potential application in other pharmacological and cosmetic industry as a test bench for safety testing of medical technologies for health, or new products for skin care, contributing, at the same time, to accelerate and simplify the pre-clinical testing of new drugs or treatments while reducing animal testing. The Food and Drug Administration (FDA), the National Institute of Health (NIH) and many scientific journals have all been playing an important role in the promotion of rigorous principles and guidelines in pre-clinical research in the United States and beyond. A strong increase in the use of alternative models to animal testing is foreseeable for coming years.

A successful implementation of the 3Rs initiative starts with a conscientious design study of the experiments and the use of alternative models to animal testing whenever possible.

We propose two developed types of bioreactors that may represent such alternative systems that can help identifying the bottleneck of *in vivo* approaches prior to moving to animal testing and thus can contribute to minimize the number of animals needed. In addition, more complex, bioreactor-based *in vitro* models (by using organs from large animals) may also help to predict the *in vivo* response and could be useful to recognise innovative pharmacological treatments.

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Materials and Methods

The dynamic stretching bioreactor

Dynamic culture experiments were carried out with a bioreactor specifically designed and previously realized. Briefly, the device is composed of a Plexiglas® culture chamber, able to house four strip samples lying parallel during culture, and a mechanical stimulation subsystem (a stepping motor, two drive shafts and four pairs of grips holding the specimens at the extremities). The device components can be sterilized in ethylene oxide, allowed to degas for 5 days and easily assembled under a laminar flow hood due to support tools specifically designed for the purpose. Figure 1 shows the bioreactor chamber. During culture, a PLC controller drives the stepping motor, enabling reliable and reproducible stretching of four engineered constructs in parallel.

The decell-bioreactor

The device is composed of a chamber to house the arterial vessel by mean of POM proximal holding and stainless steel distal holding and closed by a POM cap provided with venting access. Three reservoirs house the decellularization solution pumped inside the chamber by means of the first pump, electrovalves lead flows in the right direction, heat exchanger is positioned on the recirculating line in order to control fluid temperature according to sensor measurements, an emptying pump pulls out decellularization solutions from the chamber to the waste container, a microcontroller controls the whole system.

Swine vessels were decellularized according to a previously tested protocol using an in-house developed device for the automatic decellularization of biological tissues.[2] Briefly, the treatment consists of 4 cycles as follows: 72 h washing in deionized water with 1% AA solution at 4°C followed by 4 h in sodium deoxycholate 4% solution at room temperature (RT) and 3 h in deoxyribonuclease I 2000 kU in sodium chloride 1 M at RT.

Results

Stretching bioreactor

Figure 1a shows the biomechanical pattern superimposed by the bioreactor. As previously described, once attached to the fiber surface, C2C12 myoblasts spread out and multiplied, forming layers of cells aligning parallel to the fiber axis. The effect of the applied stretching pattern on the development of skeletal muscle engineered constructs was evaluated by investigating MHC accumulation in stretched constructs, using statically cultured samples (S) as controls. Western blot analysis on protein homogenates and immunostaining of total sarcomeric MHC, known as a marker of terminal differentiation, were performed. Figure 1b shows the result of Western blot

for MHC on dynamically (D) and statically (S) cultured samples, at DAY 0 (D0, S0), DAY 7 (D7, S7) and DAY 10 (D10, S10).[2,3]

The decell-bioreactor

Native and decellularized vessel samples were stained with hematoxylin-eosin and DAPI. The overall histological results revealed the absence of cells or nuclear matter. Hematoxylin and eosin staining showed a preserved ECM structure at the end of the decellularization process with no residual cells, confirmed by DAPI fluorescent staining.

The spectrophotometric analysis revealed that the decellularization process removed the majority of the DNA content in the treated tissue compared to the native one as shown in Figure 2. Native and decellularized vessels samples were freeze dried, gold coated, and observed with ESEM. No evidence of damage was observed after either the freezing step or the decellularization process. The mechanical testing analysis resulted in no statistically significant differences for Young's modulus, compliance, ultimate circumferential stress, burst pressure, and suture retention strength; on the other hand, there was a significant loss in ultimate strain between native and decellularized vessels; moreover, residual stress after relaxation was increased for decellularized samples compared to native ones.

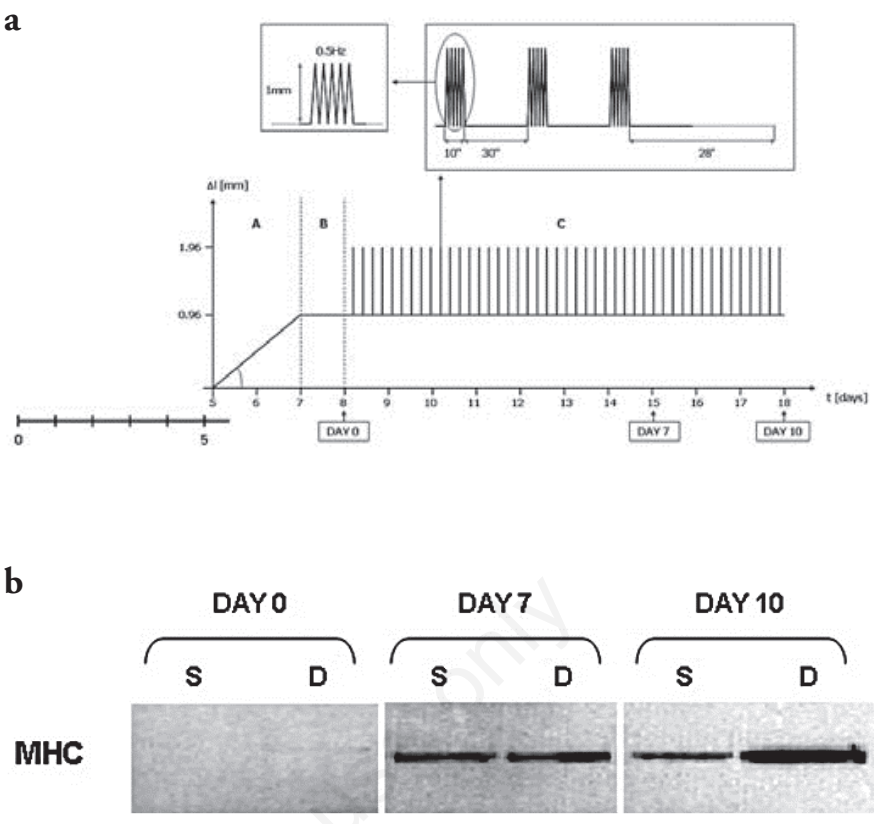

Figure 1. a) Stretching pattern applied during mechanical conditioning, adapted from the literature to myoblasts of murine origin. The unidirectional stretching phase is meant to mimic bone growth during embryonic development, while the phase of cyclic stretch resembles increasing functional demand of the developing fetus. b) Typical Western blot picture representing the accumulation of total myosin heavy chain (MHC) at days 0, 7 and 10 from the onset of cyclic stretching on stretched and control engineered constructs.

Conclusions

The bioreactors proposed are able to develop a biological construct based on synthetic scaffold and cell line (stretching bioreactor) and a de-personalized biological scaffold (decell-bioreactor).

The stretching bioreactor allows the preservation of musculoskeletal tissue architecture and function under physiological mechanical stimuli and can help to identify crucial factors involved in normal tissue development, homeostasis, injury, and pathology and can be used to screen novel treatments in a highly-relevant environment.

A very promising development is represented by the decell-Bioreactor: decellularization allows obtaining non-immunogenic scaffolds with the native structure of the processed tissue and biochemical properties that promotes cell adhesion, proliferation and differentiation. The decellularization approach has been used for various simple structure tissues, like blood vessels, trachea, bladder or skin with positive outcomes and even clinical applications. A promising evolution of this approach that is currently

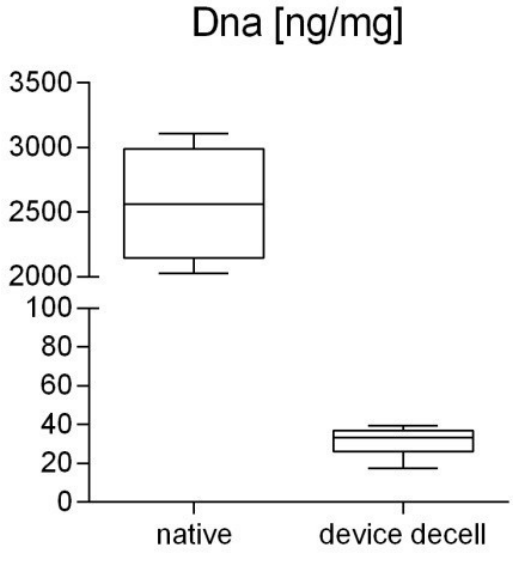

Figure 2. Residual DNA content. The quantification of the residual DNA in swine blood vessels after the decellularization process revealed a quantity of DNA far low from the native one.

being largely investigated is the decellularization of complex organs that could be used, after proper recellularization as whole organ *in vitro* model for further scientific investigation.

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