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SQPR 3.0: a sensorized bioreactor for modulating cardiac phenotype

S. Giusti[a]*, F. Pagliari[a], F. Vozzi[b], A. Tirella[a], D. Mazzei[a], M. Cabiati[b], S. Del Ry[b], A. Ahluwalia[a,b]

[a]*Research Center "E. Piaggio", University of Pisa, Via Diotisalvi 2, 56126 Pisa, Italy*
[b]*Institute of Clinical Physiology IFC-CNR, Pisa, Italy, Via Giuseppe Moruzzi 1, 56124 Pisa, Italy*

Abstract

In cardiac tissue engineering, the use of bioreactors is fundamental for applying controlled mechanical stimuli on cells and recreate a physiological environment for cardiomyocyte cultures. This work is focused on the design of a sensorized Squeeze Pressure bioreactor (SQPR 3.0) able to apply a periodic contactless hydrodynamic pressure on tissue constructs. This system was then tested with H2c9, a murine cardiomyoblast cell line, to investigate the effect of different stimulation times (2h, 24h, 30h) on cell shape and cardiotypic marker expression.

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Keywords: bioreactor; hydrodynamic pressure; force sensor; H9c2; phenotypic modulation.

* Corresponding author. Tel.: +39-050-2217061; fax: +39-050-2217051.
E-mail address: serena.giusti@centropiaggio.unipi.it

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1. Introduction

The biomechanical properties of tissues are critical to their proper function in vivo. In tissue engineering, in order to repair or replace these load-bearing structures effectively, cells must grow in an appropriate environment which recapitulates the mechanical as well as biochemical context of the physiological environment. Bioreactor systems play an important role because they can provide controlled environments for reproducible and accurate application of mechanical forces to engineered tissues and 3D constructs. In cardiac tissue engineering, the ability to recreate a more physiological environment is particularly relevant, because cardiac cells are normally subjected to mechanical stresses during hemodynamic loading and unloading [1]. These forces can modulate the physiological development and function of cells inducing modifications in cell structure, contractile function and, basically, in gene expression. Traditionally, two different stimuli are applied on cardiac cells: mechanical stimuli, such as pressure [2], flow [3], shear stress [4] and stretch [5], or electrical stimuli [6].

This work is focused on the design and testing of a sensorized Squeeze Pressure bioreactor (SQPR 3.0) able to apply a periodic contactless hydrodynamic pressures on tissue constructs thanks to the cyclic movement of a piston [7]. A previous version of this bioreactor has already been tested on different cell cultures (i.e. chondrocytes, cardiomyocytes), demonstrating the ability of the system to upregulate the rate of ECM synthesis and increase cellular organization in the scaffold. In order to apply the desired stimulus on a cell culture, SQPR 3.0 is provided with a force sensor and a position sensor, assuring high precision and control of the piston movement.

This bioreactor was then tested on the embryonic rat ventricular cell line H9c2, which is widely used as in-vitro model of cellular model for studying physiologic and pathophysiologic mechanisms in the heart [8]. In particular, the ability of the SQPR 3.0 to induce modification in cell morphology and phenotypic expression on H9c2 was investigated. Cells were seeded on collagen sandwiches and subjected to a cyclic overpressure of 1 kPa with 1 Hz frequency for 2, 24 and 30 hours. Cells were recovered for viability testing to confirm that the hydrodynamic environment present in the SQPR chamber did not compromise cell function. Moreover, the ability of the system to induce changes in cell shape and cytoskeletal organization was assessed using Phalloidin/DAPI staining. Furthermore, changes in gene expression in response to the fluid-induced forces and overpressure applied by the SQPR were evaluated measuring BNP gene expression.

2. Design of SQPR 3.0 and Cellular Experiments

2.1. The SQPR 3.0

As shown in Fig. 1, the bioreactor is composed of three main parts: a Plexiglas chamber, a base in Delrin on which an Aluminum sample brace is inserted, and a piston. The working principle is very simple and it is completely described in [7]. In brief, when the piston moves down, it creates a local overpressure on the base of the bioreactor, where the cells are placed, without touching the sample. The entity of the stimulus mainly depends on the piston velocity and the distance between the two approaching surfaces. In addition, thanks to the cyclic piston movement, the culture media flows through the cell-seeded construct enhancing the diffusion of oxygen and nutrients.

Computational FEM models of the bioreactor chamber were previously developed [7], showing that the pressure stimulation strongly depends on the distance between the piston and the stimulated construct. Therefore, to better control the applied pressure, the position of the piston has to be precisely regulated and measured. The new SQPR 3.0 is therefore provided with a force sensor (Tekscan, Inc. MA, USA), placed under the sample brace, able to detect any contact between the piston and the scaffold (Fig. 2.A).

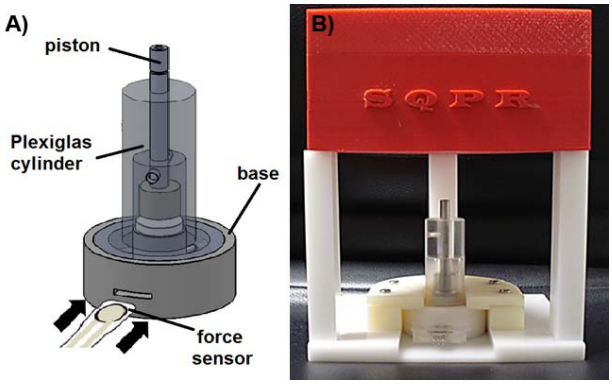

Fig. 1. A) The SQPR chamber CAD drawing realized with SolidWorksTM (Concord, Massachusetts, USA); B) SQPR chamber in its frame.

The sensitivity of the sensor was also determined by the contact area. For this reason the bottom part of the sample brace was designed so as to assure the application of the whole load on the sensing area of the sensor.

All the data from the force sensor were compared in real-time with a position sensor placed on the motor shaft which drives the piston, assuring high precision and control of the motion with an accuracy of 5 µm. Furthermore, the presence of the force sensor allows compressive tests on the stimulated construct for real-time evaluation of the matrix production, linked to the stiffness of the scaffold (Fig. 2.B).

A dedicated control unit interfaced to a PC allows setting of all stimulation parameters using an appropriate interface. The user can easily set the maximum value of the hydrodynamic pressure applied, the frequency of the stimulus and the time of the experiment. The system automatically finds the starting position of the piston, and applies the desired pressure for the established experimental time.

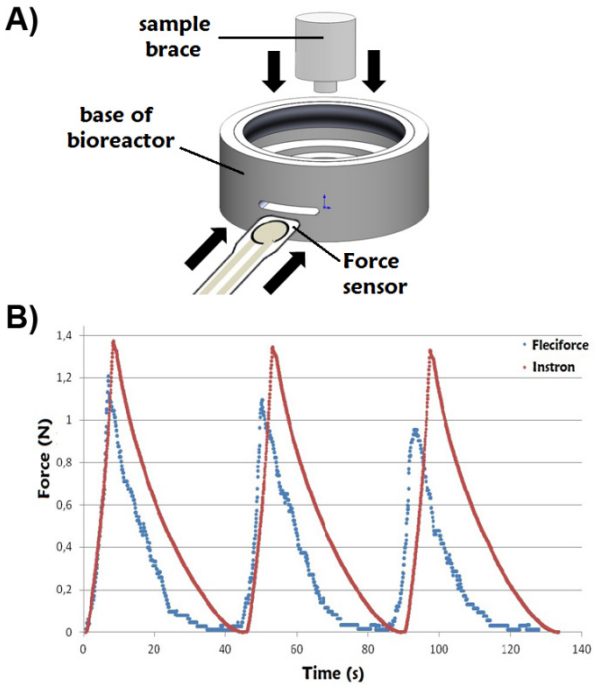

Fig. 2. (A) Schematic view of the SQPR base with the force sensor and the sample brace, specifically designed for increasing the sensitivity of the force sensor; (B) Comparison between the Flexiforce and the Instron outputs (Zwick-Roell Z005, Zwick Testing Machines Ltd., UK).

2.2. Cell culture and experimental protocol

H9c2 (ATCC, Manassas, VA, USA), a myoblast cell line obtained from rat heart tissue, were cultured in Dulbecco Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) of Fetal Bovine Serum, 4 mM-glutamine, 20 U/mL penicillin and 20 μg/mL streptomycin. Cells were seeded on 12 mm diameter glass coverslips ($6 \cdot 10^4$ cells/sample), pre-treated with a 0.3 mg/mL collagen type I solution extracted from rat tail [9], allowing protein adsorption on glass surface. After 24 hours from cell seeding, a collagen coating was added on the cell layer, thus forming a sandwich (Fig. 3), as described in the Appendix A.

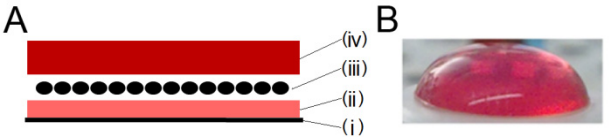

Fig. 3. A) Schematic view of the collagen sandwich construct: (i) glass coverslip Ø 12 mm, (ii) first collagen layer 0.3 mg/mL, (iii) cell in monolayer, (iv) second layer of collagen 3 mg/mL; B) 2D collagen sandwich construct.

Then, one collagen sandwich was placed on the sample brace in the bioreactor chamber and the second one was transferred into a 12-well plate, as a static control. The chamber was then closed, and 3 mL of culture media was added to the bioreactor as well as to controls. Both the bioreactor and controls were maintained at 37°C, 5% CO_2. Experiments were performed applying 1 Hz stimulation for 2, 24 and 30 hours. At the end of the experiments, the constructs were removed from the SQPR chamber by carefully withdrawing the media and unscrewing the base. Samples were transferred to culture plates and then analyzed as described in the next section.

2.3. Cellular Assay and Staining

After the stimulation in the SQPR 3.0, cell viability was assessed by using the LIVE/DEAD Assay Kit (Invitrogen, Paisley, UK), according to the manufacturer's specifications. Samples were observed with an inverted fluorescence microscope (IX81 Olympus, Germany). This assay was used to determine whether the mechanical stimulation produces localized cell damage and for performing a quantitative analysis of cell viability using ImageJ. The number of living cells was estimated by dividing the total fluorescent area of each micrograph with the average area of the cells. The number of dead cells was calculated by analyzing calcein intensity after the application of the appropriate threshold.

Then, cytoskeletal organization and cell shape after the hydrodynamic stimulus was evaluated on the same samples labelling F-actin filaments with Rhodamine Phalloidin (Molecular Probes®) and counterstaining nuclei with DAPI. Samples were fixed with 4% paraformaldehyde and incubated with 1 μg/mL DAPI for 5 min and 150 nM of rhodamine-phalloidin for 30 min, respectively and imaged using a confocal microscope (Nikon A1 Confocal Microscope System).

Finally, for evaluating the BNP mRNA expression, the total RNA was extracted as described in [10]. Following DNAse treatment (RNase-Free DNase Set, QiagenS.p.A, Milano, Italy), first strand cDNA was synthesized with iScriptcDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) using about 1 μg of total RNA as template. Reverse transcriptase reaction sequence consisted of incubation at 25°C for 5 min, followed by three different cycles at 42°C for 30 min and 45°C-48°C for 10 min, in order to better separate the strands. The reverse transcriptase enzyme was inactivated by heating to 85°C for 5 min. Then, RT-PCR reactions were performed in duplicate in the Bio-Rad C1000™ thermal cycler, as described in the Appendix B. .

2.4. Results

Results show that H9c2 viability up to 30 h of stimulation was similar to that of static controls (SQPR 30 h: 96% ± 1.3; static 30 h: 98% ± 0.8), thus indicating that mechanical stress produced on cells by SQPR did not compromise cell metabolic activity (Fig. 4).

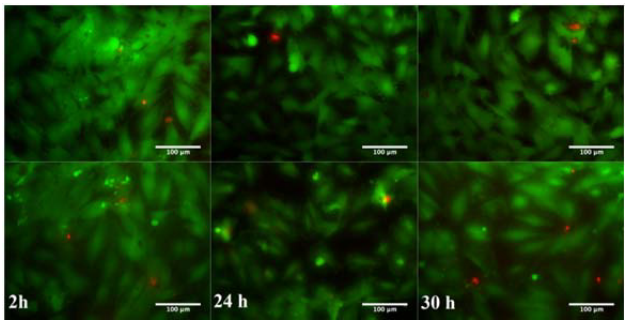

Fig. 4. LIVE/DEAD assay performed on cells after 2, 24 and 30 hours of cyclic hydrodynamic stimulation (lower line) with respecting to the static control (upper line). (scale bar = 100 µm).

Futhermore, Phalloidin/DAPI staining (Fig. 5) shows that under static condition cells are more elongated while in dynamic conditions they become increasingly cobble stone in shape. We do not observe any sign of stress or decrease in viability, nor depolymerization or changes in actin filaments.

In figure 6 we report the mean value obtained for BNP mRNA expression with RT-PCR in the control and after 2 and 24 h stimulation in the SQPR. While there was little difference in BNP expression after 2 h of stimulation, we observed a tenfold increase in BNP mRNA after 24 hours of hydrodynamic stimulus.

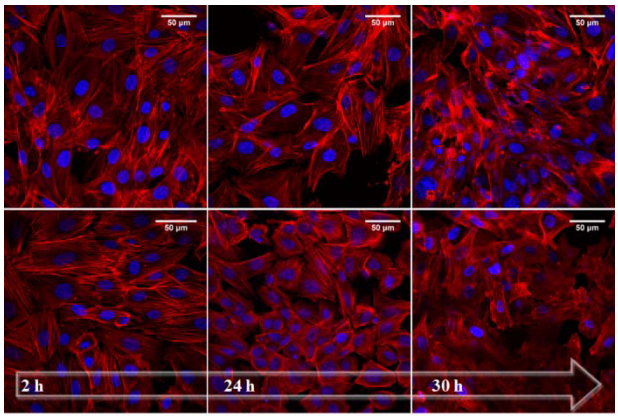

Fig. 5. Actin fibers (red, rhodamine-phalloidin probe) and nuclei (blue, DAPI probe) visualization in control (upper panel) and stimulated (lower panel) cells after 2h, 24h and 30h (20X, scale bar = 50 µm).

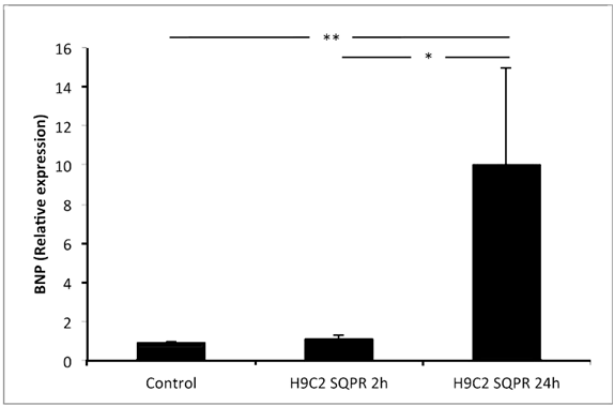

Fig. 6. BNP mRNA expression in control and treated cells in the SQPR (*p<0.01 for H9C2 SQPR 24h vs H9C2 SQPR 2h, **p<0.01 H9C2 SQPR 24h vs Control).

3. Conclusion

Our findings confirm that mechanical loading is crucial for the generation of a cardiotypic response, and that the SQPR bioreactor system is capable of applying an appropriate hemodynamic stimulus. The hydrodynamic environment in the SQPR 3.0 is particularly suitable for cardiac cells as it is contactless and can be finely controlled thanks to the high precision and control of piston movement. The new SQPR 3.0 is an easy to use standalone device for cell cultures able to perform reproducible experiments. We verified that the stimulation applied by the SQPR 3.0 bioreactor did not compromise the viability of the cells up to 30 h of cyclic stimulation. Morphological analysis also showed that the longer stimulation induced modifications in cell shape, becoming more cardiac-like shape. Finally, the SQPR stimulation induces modifications in gene expression of BNP, a cardiac-specific natriuretic peptide known to play a fundamental role in cardiovascular homeostasis. BNP mRNA expression was significantly up-regulated when cells were exposed to at least 24 h of mechanical stimulation with respect to both the controls and the 2 h squeeze pressure stimulus, indicating that the duration of the applied force is also critical for the modulation of cell response.

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Appendix A. Collagen coating

The collagen coating was prepared using a 3.0 mg/mL collagen solution in acetic acid 0.02 N. The solution was then kept in ice and M199 10X (pH 9.4) was added with 9:1 collagen:medium volume ratio in order to neutralize and promote the physical gelation of collagen (30 minute incubation at 37C). A volume of 250 μL of collagen coating solution was added to each well.

Appendix B. Real Time (RT)-PCR

PCR was performed in a volume of 20 μL per reaction. Reaction mixture included 2 μL of template cDNA [100 ng/ μL], 200 mM of each primer, 1X SsoFASTEvaGreenSuperMix (BioRad) and sterile H_2O. Amplification protocol started with 98°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Melting curves were generated from 65°C to 95°C with increments of 0.5°C/cycle. RT-PCR for the reference genes (ACTB, GAPDH, PPIA) and BNP was carried out in duplicate using the appropriate primers. Each primer sequence was designed with Primer Express Version 2.0 (Applied Biosystems) and, whenever possible, intron-spanning primers were selected to avoid amplification of genomic DNA. Reaction conditions of all primer pairs used were optimized. In particular, a gradient PCR was conducted to assess the optimal annealing temperature while a standard curve obtained by scalar dilution of a cDNA pool was always generated to verify PCR efficiency.