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Effects of the hydrostatic pressure in *in vitro* beating cardiac syncytia in terms of kinematics (kinetic energy and beat frequency) and syncytia geometrical-functional classification

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Abstract— **Many important observations and discoveries in heart physiology have been made possible using the isolated heart method of Langendorff, e.g. the discovery of the very famous Frank-Starling law of the heart. Nevertheless, the Langendorff's method has some limitations and disadvantages such as the probability of preconditioning and a high oxidative stress, leading to the deterioration of the contractile function. To avoid the preceding drawbacks associated to the use of a whole heart, we have alternatively used beating mouse cardiac syncytia cultured *in vitro* in order to assess the ergotropic and chronotropic effects of both increasing and decreasing hydrostatic pressures. To achieve the preceding aim, we have developed a method based on image processing analysis to evaluate the kinematics of that pressure-loaded beating syncytia starting from the video registration of their contraction movement. We have verified the Frank-Starling law of the heart in *in vitro* beating cardiac syncytia and we have obtained their geometrical-functional classification. The present method could be used in *in vitro* studies of beating cardiac patches, as alternative to the Langendorff's heart in biochemical, pharmacological, and physiology studies, and, especially, when the Langendorff's technique is inapplicable. Furthermore, the method could help, in heart tissue engineering and bioartificial heart researches, to "engineer the heart piece by piece".**

I. Introduction

Several important observations and discoveries in heart physiology have been made possible by the isolated heart method of Langendorff [1], where an isolated heart is perfused in retrograde manner via an aortic cannula at a constant hydrostatic pressure (pressure mode) or at a constant flow rate (flow mode). In his pioneering studies, Langendorff demonstrated that the heart receives its nutrients and oxygen from blood through the coronary arteries and that cardiac mechanical function is reflected by changes in the coronary circulation. In addition, the Langendorff's method permitted the study of cardiac metabolism and the discovery of the very famous Frank-Starling law of the heart [2]. Nevertheless, the Langendorff's method presents some limitations and disadvantages such as the vulnerability of the excised heart to contusions and injuries, the probability of preconditioning during instrumentation, the possibility to induce tissue oedema, and a high oxidative stress, leading to the deterioration of the contractile function [3].

In order to avoid the preceding drawbacks associated to the use of a whole heart, methods based on single beating heart cells [4,5] and on beating cardiac syncytia [6-13] have been developed. To extend the possible use of beating cardiac syncytia cultured *in vitro*, especially when the Langendorff's technique is inapplicable (e.g., in studies about human cardiac syncytium in physiological and pathological conditions, patient-tailored therapeutics, and syncytium models derived from induced pluripotent/embryonic stem cells with genetic mutations), in a previous work, we have developed a novel method based on image processing analysis to evaluate the kinematics of *in vitro* beating syncytia starting from the video registration of their contraction movement [14]: in particular, our method uses the displacement vector field and the velocity vector field of a beating patch to evaluate the syncytium not only from the chronotropic viewpoint, but also from the ergotropic one. In the present work, the preceding calculus method allowed to study the mechanical modulation of the contraction properties in *in vitro* beating cardiac syncytia as they were loaded with different hydrostatic pressures. In particular, the computed kinematic parameters aimed at revealing the ergotropic and chronotropic effects of the applied hydrostatic pressures: we anticipate that the data analysis permitted the verification of the Frank-Starling law in *in vitro* beating cardiac syncytia and their geometrical-functional classification.

II. Materials and Methods

A. Beating mouse cardiac syncytia

Spontaneously beating cardiac syncytia were obtained from hearts of 1- to 2-day-old CD-1® mouse pups (Charles

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River Laboratories Italia, Calco, Italy) [6-8]. Briefly, beating primary cultures of murine cardiomyocytes were prepared *in vitro* as follows: the hearts were quickly excised, the atria were cut off, and the ventricles were minced and digested by incubation with 100 μg/ml type II collagenase (Invitrogen, Carlsbad, CA) in ADS buffer (0.1 M HEPES, 0.1 M D-glucose, 0.5 M NaCl, 0.1 M KCl, 0.1 M $NaH_2PO_4 \cdot H_2O$, 0.1 M $MgSO_4$) for 15 min at 37°C and then by incubation with 900 μg/ml pancreatin (Sigma-Aldrich, Milan, Italy) in ADS buffer for 15 min at 37°C. The resulting cell suspension was preplated for 2 h at 37°C to reduce the contribution of non-myocardial cells. The unattached, cardiomyocyte-enriched cells remaining in suspension were collected, plated onto collagen-coated 35-mm Petri dishes, and covered by DMEM containing 10% horse serum, 5% fetal bovine serum, and 1× gentamicin (Roche Molecular Biochemicals, Indianapolis, IN). About 3×10^5 cardiomyocytes were cultured in each Petri dish at 37°C and 5% CO_2 to form a spontaneously beating cardiac syncytium.

B. Pressure apparatus

On day 3 of culture, at a constant temperature of 37°C and 5% CO_2, each syncytium was observed at 20× magnification via a movie capture system (ProgRes C5, Jenoptik, Germany) in four conditions of relative hydrostatic pressure: at 0 (atmospheric pressure), 100, 200, and 300 mmHg inside a custom-machined PMMA pressure chamber (Fig. 1).

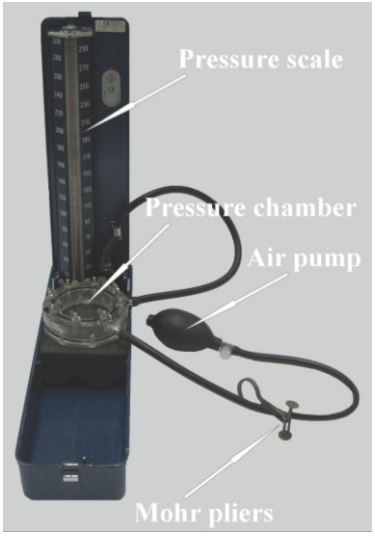

Figure 1. Pressure apparatus. A pump insufflates air into a pressure chamber and the pressure is measured via a mmHg scale.

The syncytium cultures were repeated 4 times for a total of 40 syncytia loaded by both increasing and decreasing pressures. In particular, for each syncytium, we have obtained a sequence of eight video files in AVI format, each video with a duration of 40 s: at 0, 100, 200, 300 mmHg (after that there was a recovery of 0.5 h in incubator at 37°C and 5% CO_2), then at 300, 200, 100, and 0 mmHg.

C. Registration of the syncytium movement

By the Video Spot Tracker (VST) program, which is used to track the motion of one or more spots in an AVI video file (http://cismm.cs.unc.edu/downloads), in each video, we have systematically selected 30 spots or markers onto the first video frame, according to the same orthogonal grid. By starting the videos in VST, frame by frame, the program followed and registered the spatial-temporal coordinates x, y, and t for each marker. The coordinates x and y are expressed in [pixel], whereas the coordinate t in [s].

D. Kinematics of the beating syncytium

The mathematical foundations of our method briefly follow. By an algorithm based on the Matlab programming language (The MathWorks, Inc., Natick, MA), frame by frame and for each marker, we have calculated the displacement vector $\underline{s}=\underline{s}(x,y,t)$, the velocity vector $\underline{v}=\underline{v}(x,y,t)$, and the acceleration vector $\underline{a}=\underline{a}(x,y,t)$ [14].

Being both contraction and relaxation active phases of the syncytium movement, in order to estimate a possible ergotropic effect of the pressure, that is, the metabolic consumption of a quota of the stored adenosine triphosphate (ATP), which is in part transformed in kinetic energy of the beating cells during the contraction-relaxation movement, we have defined E as the mean kinetic energy (in [joule]) of a beating syncytium in a discrete video:

$$E = \frac{1}{2} A \frac{B}{NM} \sum_{i=1}^{N} \sum_{j=1}^{M} \left| \underline{v}_{i,j} \right|^2 \tag{1}$$

where $\underline{v}_{i,j}$ is the velocity of the marker i in the frame j, M is the total number of video frames, N is the total number of markers (N=30), A is the constant related to the tissue mass, and B is the constant derived from the linear relation between the units meter and pixel in a bitmap AVI video at a given magnification (magnification=20×).

According to (1), for each syncytium, in order to compare the pressure effects, there was no need to know the mass of the beating tissue or the A constant, because that mass and constant were the same in the four different conditions of relative hydrostatic pressure and the spot markers were juxtaposed in the same grid positions. In addition, there was no need to know the video metrics or the B constant, because that metrics and constant and the video magnification were the same at all pressures. As consequence, we have defined E_{norm} as the normalized mean kinetic energy (in [$pixel^2/s^2$]) of a beating syncytium in a discrete video:

$$E_{norm} = \frac{E}{\frac{1}{2} AB} = \frac{1}{NM} \sum_{i=1}^{N} \sum_{j=1}^{M} \left| \underline{v}_{i,j} \right|^2 \tag{2}$$

Besides, for each marker i, in order to calculate B_i as the total number of beats, we have identified and counted the peak displacements, that is, the peak Δx and Δy of the contractions [14]. Given the duration T of the video (T=40 s), we have defined f as the mean beat frequency (in [Hz]):

$$f = \frac{1}{TN} \sum_{i=1}^{N} B_i \tag{3}$$

E. Statistics

In order to compare the results between the different pressure conditions, one-way Analysis of Variance (ANOVA) with *post hoc* Least Significance Difference (LSD) test was applied, electing a significance level of 0.05. The results are expressed as mean ± standard deviation.

III. Results

The software and calculus method previously described allowed to study the mechanical modulation of the contraction properties in *in vitro* beating cardiac syncytia as they were loaded with different hydrostatic pressures. In particular, the computed kinematic parameters aimed at revealing possible ergotropic and chronotropic effects of the applied hydrostatic pressures.

A. Geometrical-functional classification of the syncytia

We observed particularly thick multilayers with "spheroidal" shape and "flat" multilayers (Figs. 2 and 3), and we evaluated them in terms of kinetic energy (E_{norm}) and beat frequency (f).

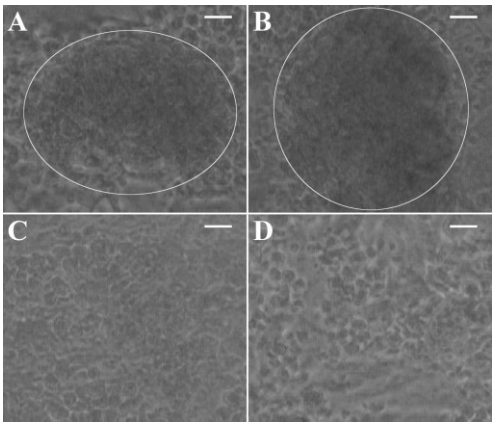

Figure 2. Syncytia. Cellular layers in beating syncytia (bars equal to 50 µm, 20× magnification). We observed particularly thick multilayers with "spheroidal" shape (*A-B, white ellipses*) and "flat" multilayers (*C-D*).

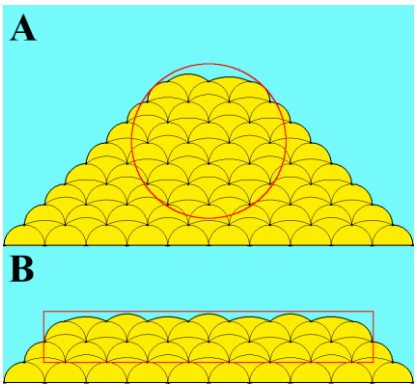

Figure 3. Syncytia. Scheme of cellular layers in beating syncytia: transversal section of a thick multilayer with "spheroidal" shape (*A, red circle*) and transversal section of a "flat" multilayer (*B, red box*).

The spheroidal syncytia showed minimum kinetic energy and maximum beat frequency at 200 mmHg, with $p<0.05$ in the comparison with the corresponding adjacent values at 100 and 300 mmHg (Figs. 4 and 5), except for the kinetic energy ($p>0.05$) (Fig. 4).

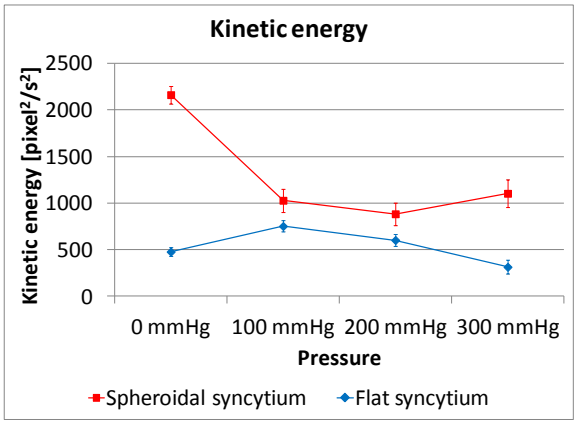

Figure 4. Kinetic energy. The spheroidal syncytia showed minimum kinetic energy at 200 mmHg (without statistically significant differences in comparison with the adjacent values at 100 and 300 mmHg [$p>0.05$]); the flat syncytia were characterized by maximum kinetic energy at 100 mmHg (with statistically significant differences in comparison with the adjacent values at 0 and 200 mmHg [$p<0.05$]). At all pressures, the kinetic energy of spheroidal syncytia was significantly higher than that of flat ($p<0.05$).

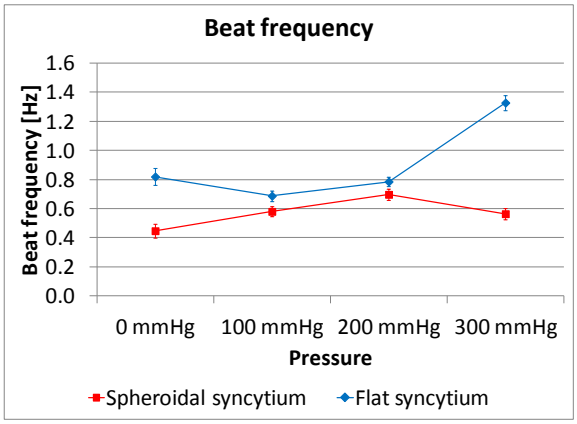

Figure 5. Beat frequency. The spheroidal syncytia showed maximum beat frequency at 200 mmHg (with statistically significant differences in comparison with the adjacent values at 100 and 300 mmHg [$p<0.05$]); the flat syncytia were characterized by minimum beat frequency at 100 mmHg (with statistically significant differences in comparison with the adjacent values at 0 and 200 mmHg [$p<0.05$]). At all pressures, the beat frequency of spheroidal syncytia was significantly lower than that of flat ($p<0.05$).

The flat syncytia were characterized by maximum kinetic energy and minimum beat frequency at 100 mmHg, with $p<0.05$ in the comparison with the corresponding adjacent values at 0 and 200 mmHg (Figs. 4 and 5).

At all pressures, the kinetic energy of spheroidal syncytia was significantly higher than those of flat syncytia ($p<0.05$) (Fig. 4). In addition, at all pressures, the beat frequency of spheroidal syncytia was significantly lower than the beat frequency of flat syncytia ($p<0.05$) (Fig. 5).

IV. Discussion

The mouse has become a cornerstone of the heart research because of the high potential in manipulating its genome and the consequent availability of models of cardio-

vascular diseases. Using beating primary cultures of murine cardiomyocytes, we have verified the Frank-Starling law of the heart according to the following terms.

In the spheroidal syncytia, where a better 3D distribution of pressure loads was possible in comparison to flat syncytia (Fig. 3), an increasing pressure theoretically caused a shortening of the sarcomeres with consequent decreased ergotropy and increased chronotropy (in the 0÷200 mmHg pressure range, Figs. 4 and 5); on the other hand, in the flat syncytia, where the increasing pressure loads uniformly spread onto a 2D surface (Fig. 3), the sarcomeres were theoretically stretched with consequent increased ergotropy and decreased chronotropy (in the 0÷100 mmHg pressure range, Figs. 4 and 5).

The preceding trends were then inverted for pressures higher than 100 mmHg and 200 mmHg in flat and spheroidal syncytia, respectively. We hypothesize the presence of overstretched sarcomeres in the flat and thin syncytia already at low pressures (>100 mmHg) with consequent impairment of the contractile function. On the other hand, in the spheroidal and thick syncytia, where a better 3D distribution of pressure loads was possible in comparison to the flat and thin syncytia, we hypothesize increasingly packed myofibrils i.e. increasingly cross-linked actin and myosin filaments only at high pressures (>200 mmHg) with consequent amelioration of the contractile function.

In addition, in comparison to the flat and thin syncytia, the spheroidal and thick syncytia showed, at each pressure, higher ergotropy and lower chronotropy (Figs. 4 and 5): this result was consistent with the general relationship between the contractile parameters and the heart size in mammalians, where the chronotropy decreases due to increasing heart size, whereas the ergotropy increases together with the heart size [15].

V. Conclusion

In conclusion, the present method permitted a systematic study of *in vitro* beating syncytia, which were previously described in terms of cardiac markers and functional gap junctions [16]. As consequence, it could be used in *in vitro* studies of beating cardiac patches, as alternative to the Langendorff's heart in biochemical, pharmacological, and physiology studies, and, especially, when the Langendorff's technique is inapplicable (e.g., in studies about human cardiac syncytium in physiological and pathological conditions, patient-tailored therapeutics, and syncytium models derived from induced pluripotent/embryonic stem cells with genetic mutations).

Furthermore, the method could help, in heart tissue engineering and bioartificial heart researches, to "engineer the heart piece by piece" [17]. In particular, our method could be useful in: i) the identification of a suitable cell source, preferably adult-derived autologous stem cells, ii) the development of biomaterials, and iii) the design of novel bioreactors and microperfusion systems.

Ethics Statement

All procedures involving mice were completed in accordance with the policy of the Italian National Institute of Health (Protocol nr. 118/99-A) and with the ethical guidelines for animal care of the European Community Council (Directive nr. 86/609/ECC).

CD-1® mice were obtained from the Charles River Laboratories Italia (Calco, Italy) and were housed under 12-h light/dark cycles, at constant temperature, and with food and water *ad libitum*. The mice were sacrificed by CO_2 asphyxiation.

References

[1] O. Langendorff, "Untersuchungen am überlebenden Säugetierherzen," *Pflügers Archiv*, vol. 61, pp. 291-332, 1898.

[2] E. H. Starling and M. B. Visscher, "The regulation of the energy output of the heart," *J Physiol*, vol. 62, pp. 243-261, 1927.

[3] M. Skrzypiec-Spring, B. Grotthus, A. Szelag et al., "Isolated heart perfusion according to Langendorff - Still viable in the new millennium," *J Pharmacol Toxicol Methods*, vol. 55, pp. 113-126, 2007.

[4] H. G. Glitsch, "Electrophysiology of the sodium-potassium-ATPase in cardiac cells," *Physiol Rev*, vol. 81, pp. 1791-1826, 2001.

[5] T. Shioya, "A simple technique for isolating healthy heart cells from mouse models," *J Physiol Sci*, vol. 57, pp. 327-335, 2007.

[6] E. Devic, Y. Xiang, D. Gould et al., "β-adrenergic receptor subtype-specific signaling in cardiac myocytes from β_1 and β_2 adrenoceptor knockout mice," *Mol Pharmacol*, vol. 60, pp. 577-583, 2001.

[7] Y. Xiang, V. O. Rybin, S. F. Steinberg et al., "Caveolar localization dictates physiologic signaling of β_2-adrenoceptors in neonatal cardiac myocytes," *J Biol Chem*, vol. 277, pp. 34280-34286, 2002.

[8] Y. Xiang, F. Naro, M. Zoudilova et al., "Phosphodiesterase 4D is required for β_2 adrenoceptor subtype-specific signaling in cardiac myocytes," *Proc Natl Acad Sci USA*, vol. 102, pp. 909-914, 2005.

[9] O. G. Shcherbakova, C. M. Hurt, Y. Xiang et al., "Organization of β-adrenoceptor signaling compartments by sympathetic innervation of cardiac myocytes," *J Cell Biol*, vol. 176, pp. 521-533, 2007.

[10] V. De Arcangelis, D. Soto, and Y. Xiang, "Phosphodiesterase 4 and phosphatase 2A differentially regulate cAMP/protein kinase A signaling for cardiac myocyte contraction under stimulation of β_1 adrenergic receptor," *Mol Pharmacol*, vol. 74, pp. 1453-1462, 2008.

[11] D. Soto, V. De Arcangelis, J. Zhang et al., "Dynamic protein kinase A activities induced by β-adrenoceptors dictate signaling propagation for substrate phosphorylation and myocyte contraction," *Circ Res*, vol. 104, pp. 770-779, 2009.

[12] V. De Arcangelis, R. Liu, D. Soto et al., "Differential association of phosphodiesterase 4D isoforms with β_2-adrenoceptor in cardiac myocytes," *J Biol Chem*, vol. 284, pp. 33824-33832, 2009.

[13] V. De Arcangelis, S. Liu, D. Zhang et al., "Equilibrium between adenylyl cyclase and phosphodiesterase patterns adrenergic agonist dose-dependent spatiotemporal cAMP/PKA activities in cardiomyocytes," *Mol Pharmacol*, 2010.

[14] L. Fassina, A. Di Grazia, F. Naro et al., "Video evaluation of the kinematics and dynamics of the beating cardiac syncytium: an alternative to the Langendorff method," *Int J Artif Organs*, vol. 34, pp. 546-558, 2011.

[15] K. Schmidt-Nielsen, *Animal physiology: adaptation and environment*. Cambridge, England: Cambridge University Press, 1997.

[16] L. Formigli, F. Francini, S. Nistri et al., "Skeletal myoblasts overexpressing relaxin improve differentiation and communication of primary murine cardiomyocyte cell cultures," *J Mol Cell Cardiol*, vol. 47, pp. 335-345, 2009.

[17] L. Hecker and R. K. Birla, "Engineering the heart piece by piece: state of the art in cardiac tissue engineering," *Regen Med*, vol. 2, pp. 125-144, 2007.