Lonza

Cryopreserved Neonatal Rat Cardiac Myocytes

Culture Display Hypertrophy to Non-hemodynamic Factors: New Research and Development Opportunities

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Introduction

Lonza's cryopreserved Neonatal Rat Ventricular Cardiac Myocytes can be easily thawed and cultured to give physiologically normal *in vitro* cardiac cell test beds, ideal for a spectrum of assays.

Freshly prepared Neonatal Rat Ventricular Cardiac Myocytes maintained in culture are viable test beds for examining questions of cardiac cell connectivity, activity and pharmacology.[1] Furthermore, modeling cardiac toxicity, hypertrophy, anoxic and ischemic states, cellular growth and function have been well studied in cardiac cell culture preparations.[2–5, 9, 11]

We recently demonstrated that Neonatal Rat Ventricular Cardiac Myocytes can be cryopreserved, stored frozen and then thawed for cell culture application. The thawed cells display normal morphology and physiological viability in long-term cell culture. Typically, within 24 hours of thaw and plating, the cardiac myocytes spontaneously beat, with syncytial beating of the entire culture evident by 48 hours. These characteristics remain consistent across 6-well to 96-well plate culture formats. The cardiac myocytes display robust electrical and contractile activity with normal pharmacology to cardioactive and gap-junctional drug challenge. Activity and gap-junctional connectivity of the cardiac myocytes is sustained for more than 40 days in culture.

The availability of high quality, batch tested, cryopreserved Neonatal Rat Ventricular Cardiac Myocytes from Lonza, which can be simply thawed and cultured, represents a significant advantage for the laboratory, eliminating animal handling/dissection and difficult cardiac tissue preparation. In addition, the ability to control when and where the cardiac myocyte culture can be undertaken streamlines the research workflow and speeds up R&D programs.

Here, we report the results of further study of cryopreserved Neonatal Rat Ventricular Cardiac Myocytes to determine their responsiveness in culture to challenge with positive chronotropic compounds, as well as determination of whether myocardial hypertrophy can be evaluated using cultures prepared with cryopreserved cardiac myocytes. The ability of freshly cultured Neonatal Rat Ventricular Cardiac Myocytes to display hypertrophy following challenge with non-hemodynamic factors is known.[7, 10] Therefore, we chose to study non-hemodynamic hypertrophy using Angiotensin II and Isoproterenol.

Materials and Methods

Cardiac Myocyte Cell Culture

All experiments were conducted with cardiac cell cultures established using vials of cryopreserved Neonatal Rat Ventricular Cardiac Myocytes (P1-3), purified to ≥85%; Lonza Cat. No.: R-CM-561). Cells were thawed following the manufacturer's protocol, then DMEM/F12 + FBS + HS + Pen/Strep + Hepes culture medium was added and cells plated according to the requirements of each experimental protocol. For all experiments, the cultures were changed to fresh medium 4 hours after plating and then again at 2 days *in vitro*, with media changes every 2 days thereafter. Unless otherwise stated, all cultures were maintained with BrdU in the culture medium to minimize fibroblast proliferation.

Morphology

For morphological assessment, the thawed cardiac myocytes were plated on coated coverslips in 24-well plates at 5×10^5 cells/well or in 24-well plates without coverslips for up to 14 days. Cultures were fixed and stained for immunohistochemical examination of the sarcomeric protein α-actinin (anti-α-actinin, Sigma), the gap-junction connexin 43 (anti-Cx43, Zymed Laboratories), and the nuclei stain, Hoechst (Sigma).

Functional Assessment Using Multielectrode Arrays

Vials of cryopreserved cardiac myocytes were prepared for culture as described above and plated on 60-electrode format Multi Electrode Arrays (MEA; Multi Channels Systems, Reutlingen, Germany) at a concentration of 1.5×10^6 cells/MEA. Cell beating (beats/minute) was recorded by observation of the individual cultures. The cultures were also analyzed daily for spontaneous spike activity and individual network properties in long term culture (up to 7 days). Separate MEA cultures were used to assess cardiac myocyte network responsiveness to the positive chronotropic agent, Angiotensin II (Ang II). Recordings were obtained using a MEA60 System (Multi Channel Systems, Reutlingen, Germany). Recordings were performed at 37°C and signals were simultaneously sampled at 25 kHz, visualized and stored using the software MCRack (Multi Channel Systems, Reutlingen, Germany). Files were converted into Axoscope Binary File format and displayed using Axoscope software (Molecular Devices). The data were graphed as mean ± SEM.

Cell Growth and Hypertrophy

Cardiac myocytes were thawed and plated onto 6-well plates. The cell growth medium employed for our studies contains 7.5% (v/v) FBS and 7.5% (v/v) horse serum. Sera is known to cause increased cardiac cell size.[7] Therefore, we examined whether this effect was evident in our cultures. To determine the effect of serum, we compared the cell size of cardiac myocytes at Day 1 vs Day 12 in culture. Briefly, 5 mL of a cell suspension of thawed cardiac myocytes was plated into each of 3-wells (2×10^6 cells/well) of a 6-well plate. At different time points during culture, wells were trypsinized using a standard protocol. A 10 µL suspension of trypsinized cells was added onto each glass coverslip and covered with a microruled Cellattice™ Slide (Nexcelom Bioscience). Cellattice™ is an optically smooth plastic with microscopic identification and measurement markers. The combination of numbers, letters, and tick marks identifies each 25 µm in a 10 × 10 mm area of the coverslip. This allows cell size to be easily measured, with a high degree of accuracy. Photomicrographs of the plated cells were used for measurement of the cells from the microruled grids.

In parallel experiments, cryopreserved cardiac myocytes were thawed and a 1 mL suspension (4×10^5 cells/mL) was plated in each of 3-wells of a 24-well plate. These cultures were used to test for hypertrophy following treatment with Ang II or Isoproterenol. The cultures were maintained for 7 days using our standard growth medium (containing sera). The first medium change (95%) was performed 4 hours after plating, using fresh medium with 200 µM BrdU. On Day 3, the medium was replaced completely with fresh medium without BrdU. On Day 5, a 50% medium change was performed using fresh medium (without BrdU) and drug (100 nM Ang II or 1 µM Isoproterenol) was added. Control cultures were maintained in medium without drug and without BrdU. On Day 7, the cultures were fixed and stained with the monoclonal antibody, anti-α-actinin (Sigma). Data analysis: 5 – 7 images (32X) for each of the conditions (control; Ang II; Isoproterenol) were taken. The images were then processed using AxioVision software and individual cells were contoured (5–12 cells per condition) to determine the cell surface area. Measurements were averaged, normalized to control cell average surface area and graphed as mean ± SEM.
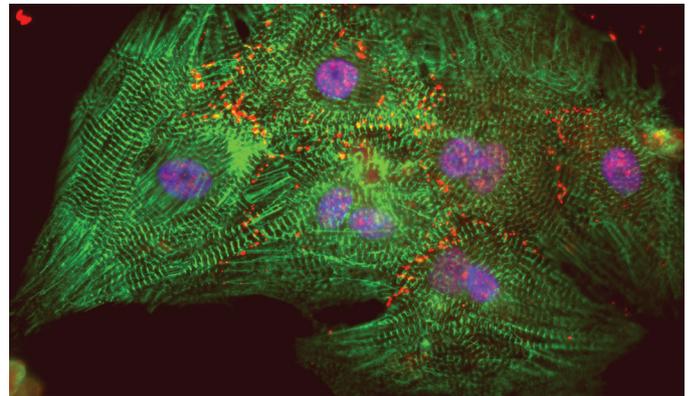

Figure 1.
Cardiac myocyte morphology and gap-junction localization. Cryopreserved Neonatal Rat Ventricular Cardiac Myocytes can be easily thawed and plated to give robust long-term cardiac cell cultures. In this high magnification micrograph of cardiac myocytes at 7 days in culture, typical anti-α-actinin staining (green) of the sarcomeres is clearly evident. The gap junctions which are critical for the two-dimensional cardiac myocyte syncytium are revealed by the bright red immunostaining of the gap-junction protein, connexin 43, localized as puncta at the interfaces of the cardiac myocytes. Nuclei (purple) are stained with Hoechst.

Results

The cryopreserved Neonatal Rat Ventricular Cardiac Myocytes thrived in culture and displayed normal morphology and gap-junction localization, as shown in Figure 1. Contractile (beating) activity was evident by 24 hours and robust physiological connectivity was apparent by observation of the whole culture beating as a syncytium within 48 hours (not shown here). This synchronous beating was reflected in the MEA recordings of electrical activity which showed the activity time-locked (synchronous) across all electrodes. Spike activity and beating synchrony was
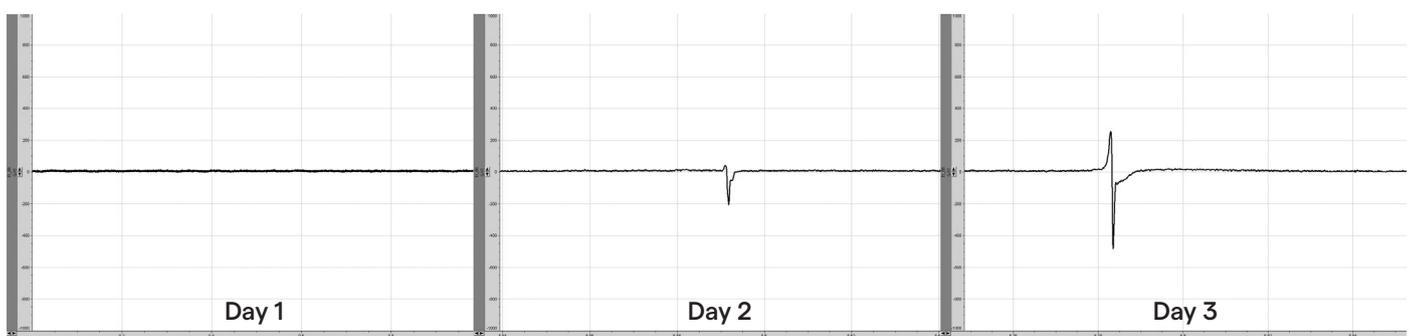

Figure 2.
MEA recording of cardiac myocyte culture electrical activity. The three-frame panel represents recordings from the same single electrode of a 64-electrode array-based cardiac myocyte culture. Observed from left to right, the frames represent Days 1 – 3 respectively. The negative and positive components of the spike potential progressively increase in amplitude.

maintained in long-term culture. The cultures displayed electrical activity from Day 1, as shown in the MEA electrode output panel of Figure 2. In this example recording from a single electrode of the MEA, the field potential recorded Day 1 through Day 3 shows a typical progressive increase in spike amplitude.

Recordings of electrical activity (which correlated with contraction frequency) across all electrodes, were measured daily for 4 days and again at Day 7 (Figure 3). By Day 2, activity frequency was 100.5 ± 13.5, rising significantly to a peak of 157 ± 28 at Day 3. At Day 4, the activity recovered to the Day 2 level. At Day 7, activity was lower but not significantly different from Day 4.

To determine whether these cultures were responsive to chronotropic stimulation, we tested Ang II and Isoproterenol. The graphed data of Figure 4 shows the increase in frequency above baseline with application of different concentrations of Ang II and Isoproterenol. This action was concentration dependent.
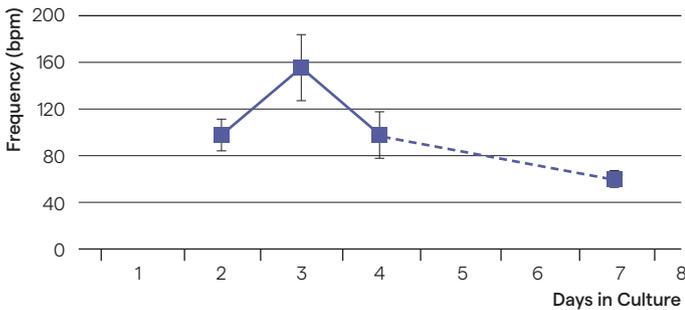

Figure 4.
Concentration dependent actions of Ang II and Isoproterenol on cardiac myocyte electrical activity. Electrical activity (correlating contractile activity) was summated and averaged across all 64 electrodes of the MEA-based cultures with and without exposure to drug.

Figure 3.
Frequency of cardiac myocyte activity in long term culture. Electrical activity (correlating contractile activity) was summated and averaged daily across all 64 electrodes of the MEA-based cultures.
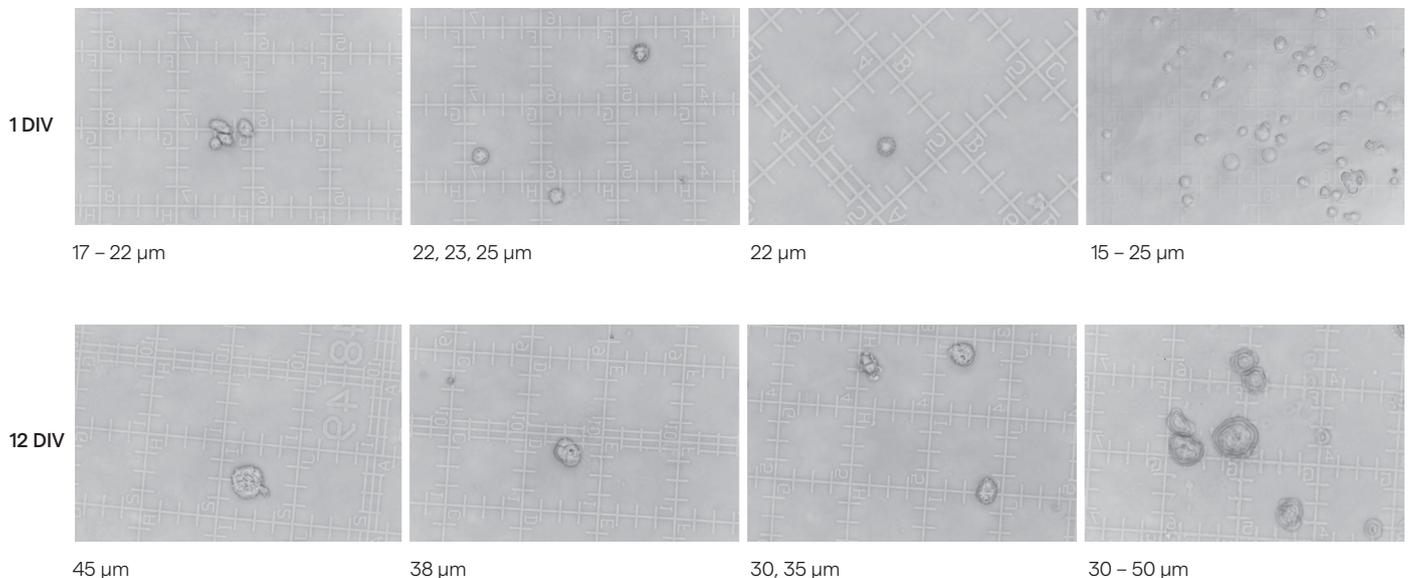

1 DIV			
17 – 22 µm	22, 23, 25 µm	22 µm	15 – 25 µm

12 DIV			
45 µm	38 µm	30, 35 µm	30 – 50 µm

Figure 5.
Cardiac myocyte growth in culture medium containing serum. Five sets of micrographs of cardiac myocytes trypsinized at Day 1 (top panel) and at Day 12 (bottom panel) can be seen in relief against the microruled grid. Each micrograph shows representative populations of cell sizes as indicated. At Day 12, the cardiac myocytes showed a large increase in average size.
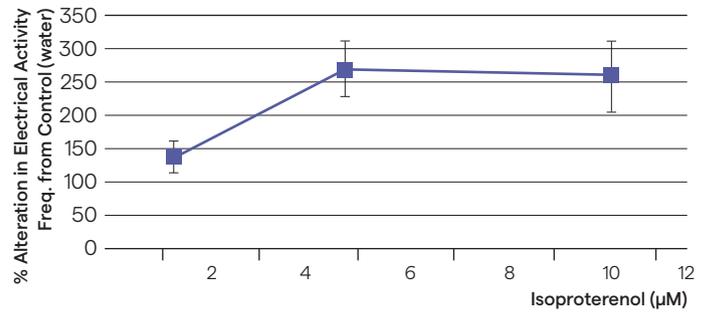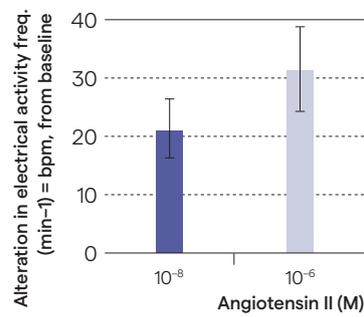
Hypertrophy

Studies using Lonza's freshly prepared Neonatal Rat Ventricular Cardiac Myocyte Cultures[7] report that cardiac myocyte hypertrophy (increased cell size) can be induced by regulating the amount of serum in the culture medium. We observed similar effects of serum in the cryopreserved Neonatal Rat Ventricular Cardiac Myocytes grown in standard medium containing serum. These cells display increased size with time in culture (Figure 5). The diameter of the trypsinized cardiac myocytes can be easily determined from the microruled surface which is clearly visible in the two sets of images. By Day 12 (bottom panel), the average cell diameter increased by 55% to 38 µm compared to the Day 1 (top panel) average of 21 µm. For the study of drug-induced hypertrophy, we used the same medium conditions across control and treatment cultures, thereby controlling for any serum effect on cell size. The ability to undergo hypertrophy was examined by exposing cardiac myocyte cultures maintained in standard medium, to either Ang II [100 nM] or Isoproterenol [1 µM] (Figure 6). Cultures were challenged with drug at Day 5 and the cultures left until Day 7, when they were washed, fixed and immunostained with anti-α-actinin. The cultures were then examined microscopically to allow surface area calculation using image software. At these concentrations, the positive chronotropic agents were comparable (50% and 58% respectively) in their ability to induce hypertrophy.
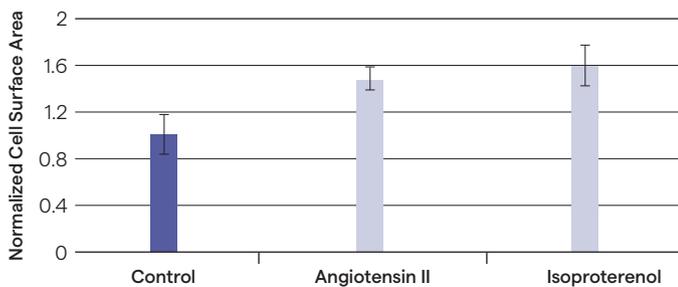

Figure 6.
Chronotropic drug treatment induces hypertrophy in cultured cardiac myocytes. The cardiac myocyte culture was exposed to either Ang II or Isoproterenol at Day 5 and the drug remained in the medium until Day 7 when the cultures were washed, fixed and immunostained to determine cell surface area by image analysis. Data were normalized and compared to untreated cultures (control) run in parallel.

Discussion

Cryopreserved Neonatal Rat Ventricular Cardiac Myocytes from Lonza are ideal for developing primary cell cultures for analysis of structure and function. In this study, we confirm and extend our previous report on the sensitivity of these cultures to pharmacological challenge and show that the profile of development of electrical activity and connectivity in these cultures is consistent with that seen in cultures of freshly prepared Neonatal Rat Ventricular Cardiac Myocytes reported by Rothermel et al. (2005).[8] In their multi electrode array studies, they observed the peak amplitude of the spike potentials to increase in intensity in long-term culture up to 5 days. We observed a similar profile in spike potential amplitude. We also found the electrical activity, which is correlated with contractions, peaked at Day 3 and by Day 7, returned to a lower stable rate. This profile of activity is comparable to that reported by Rothermel et al. (2005).[8]

Our cardiac myocyte cultures also responded with a similar increase and concentration dependence in activity (contractions) to challenge with Ang II.[8] We also found our cultured cardiac myocytes to display a concentration-dependent increase in activity to Isoproterenol, comparable to that reported by Rothermel et al. (2005).[8] Taken together with our previous report of MEA-based cultures of cryopreserved cardiac myocytes displaying concentration dependent responses to cholinergic and adrenergic drugs, these results show that cryopreservation has not affected the spontaneous contractile capacity and chronotropic sensitivity of the neonatal rat ventricular cardiac myocytes.

Further evidence that cryopreserved cardiac myocytes behave normally in culture was demonstrated when we examined the cell growth profile and hypertrophic sensitivity to long-term exposure to Ang II or Isoproterenol. These chronotropic agents are known to cause hypertrophy in freshly prepared neonatal rat heart cultures.[7, 10] In our study, treatment of rat heart cultures prepared using cryopreserved neonatal ventricular cardiac myocytes, with the same concentration of Ang II (100 nM) and for the same duration as that used by Nakamura et al.[10], caused a similar level of cardiac myocyte enlargement. We also found Isoproterenol (at the same concentration used by Simpson et al., 1982[7]) caused hypertrophy and at a level almost equivalent to that obtained with Ang II.

These results reinforce the proposal that cryopreserved Neonatal Rat Ventricular Cardiac Myocytes from Lonza display normal structure, connectivity, sensitivity and physiology when thawed and grown in long-term culture. The ease of use of these cardiac myocytes for extracellular electrophysiological examination in multielectrode array cultures ('cardiac chip') provides the researcher with an excellent cardiac screening assay. Also, the similarity in sensitivity and profile to induction of hypertrophy compared to freshly prepared cardiac myocyte cultures broadens the scope of the application of these cells in the study of cardiac function in health and disease.

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