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Cryopreserved Neonatal Rat Ventricular Cardiomyocytes

Long-term Culture Display Normal Morphology and Functional Activity

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Introduction

Cultures of cryopreserved neonatal rat ventricular cardiomyocytes thrive in long-term culture and are viable test beds for examining questions of cardiac cell connectivity, activity and pharmacology.

Isolated mammalian cardiac cells maintained in primary culture are one of the most commonly employed *in vitro* test beds for study of cardiac physiology/pharmacology.[1] Cultivated cardiomyocytes afford flexibility in the spectrum of assays that can be employed including measurement of contractility, drug transport and toxicity, screening for cardioprotective compounds, cardiac hypertrophy, analysis of anoxic and ischemic states on cellular function, as well as free radical damage and apoptosis.[2–6, 8, 10] Neonatal cardiomyocytes do not have to be made calcium tolerant during isolation[7] and cultured neonatal cardiomyocytes maintain a stable phenotype with a contractile profile during hypoxia-reoxygenation comparable to hearts *in vivo* during ischemia-reperfusion. By contrast, isolated adult cardiomyocytes have a different phenotype from that seen *in vivo*.[11]

From a use perspective, rats are one of the best described species and neonatal rat cardiomyocytes permit the study of many of the morphological, biochemical and electrophysiological characteristics of the heart.[1] Spontaneously beating cultured neonatal rat cardiomyocytes are also a very useful model to investigate the action of autoantibodies.[9] However, preparing primary cardiac cells is labor intensive and the researcher must endure the tedium, caprice and waste associated with isolating cardiomyocytes first hand. These difficulties are overcome by the introduction of cryopreserved cardiomyocytes obtained from neonatal rat hearts, available from Lonza. The neonatal cardiomyocytes are dissected and isolated in the native state from the ventricles of Sprague Dawley rats (postnatal Day 1 – 3). The pooled ventricular cardiomyocytes are purified to reduce fibroblasts, then cryopreserved in vials and stored in liquid N2.

Shipped on dry ice to the researcher, they can be simply thawed and cultured to obtain high quality and high yield cultures of dissociated ventricular cardiomyocytes. This represents a significant advantage for those interested in drug screening and cardiac pharmacology/physiology and speeds the drug development process.

Having cryopreserved cardiac cells available 'on demand' or stored ready in liquid N2 frees researchers to focus on experiments, eliminates labour intensive cell preparations from tissues, reduces the exposure of research staff to animal contact and the reduced animal use also has immense benefits for laboratory/institutional facilities. Another significant advantage over freshly dissociated cells is that collaborators from different sites can now share cardiac cells from the same batch. Cryopreserved cardiac cells from a specific shipment can also be archived in liquid nitrogen and researchers can revisit past experiments using cardiac cells from the same batch.

The results of testing cryopreserved neonatal rat ventricular cardiomyocytes for up to 47 days in culture in a variety of single and multi-well formats and on microelectrode arrays (MEAs) are reported here. MEA allow for long-term studies and multiple experiments on the same cultures. They also provide a method to analyze the detailed electrical activity of a network of cells *in vitro,* as well as network communication analysis to study the basis of synchronous activity. Therefore, we used cardiac MEAs prepared using cryopreserved neonatal rat ventricular cardiomyocytes to investigate development, syncytial network mechanisms and network responses to cardioactive drugs.

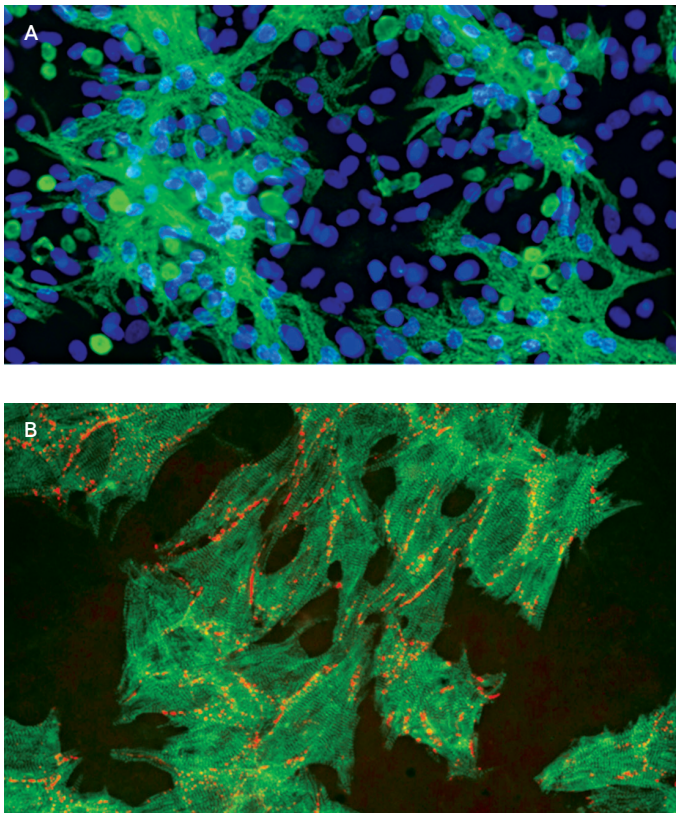

Figure 1.
Cryopreserved cardiomyocytes retain physical cardiomyocyte features. Cryopreserved cardiomyocytes establish cell-cell contacts, forming a two-dimensional syncytium of synchronously beating cells within 24 hours. This syncytium can be seen in the low (A) and high magnification (B) images of cardiomyocytes cultured 13 days without BrdU and stained for anti-α-actinin (green). The cell-cell electrotonic contacts are evidenced by immunoreactivity for the gap-junction protein Cx43 (red). Nuclei are stained with Hoechst (blue). Atrial cultures (not shown) gave similar results but were immunoreactive for Cx43 and Cx40.

Methods

We established cardiac cell cultures using vials of cryopreserved neonatal rat (P1-3) ventricular cardiomyocytes purified to 85 – 90%. Cells were thawed (2.5 minutes at 37°C), DMEM/F12 + FBS + HS + Pen/Strep + Hepes culture medium added and cells were either plated on coated coverslips in 24-well plates at 500 K per well, or plated on MEA (from Multi Channels Systems, Reutlingen Germany) at 1.5 million cells per well. The media was changed after 4 hours and again at 2 days *in vitro* (80% media changes every 2 days thereafter). Unless otherwise stated, all cultures were maintained with BrdU in the culture medium to minimize fibroblast proliferation. For morphological assessment, cultured cells on cover slips or in 24-well plates were fixed and stained for immunohistochemical examination of the protein α-actinin (located at the sarcomeric assembly) and the gap-junction connexin 43 (Cx43). MEA recordings were made at comparable time points.

Cardiomyocytes plated on 60-electrode format MEAs ('Cardiac MEAs') were treated with various cardioactive drugs: 10 µM isoproterenol, 100 µM carbachol, and 0.5 mM heptanol. For functional assessment, cell beating was noted and recorded by observation of the individual cultures. Cardiac MEAs were analyzed for spontaneous spike activity and individual network properties to characterize the effect of cardioactive substances. Recordings were obtained using a MEA60 System (Multi Channel Systems, Reutlingen, Germany) performed at 37°C and the signals were simultaneously sampled at 25 kHz, visualized and stored using the software MCRack (Multi Channel Systems, Reutlingen, Germany). Analysis was performed offline by custom-built software (Result, Tonisvorst, Germany). Files were converted into Axoscope Binary File format and displayed using Axoscope software (Molecular Devices).

Results

When thawed and cultured, the cryopreserved neonatal rat ventricular cardiomyocytes displayed >80% viability. At intervals from 1 week to 5 weeks the cardiomyocyte cultures were fixed and immunostained for α-actinin and connexin 43 (Cx43). The cryopreserved neonatal rat ventricular cardiomyocytes displayed excellent morphology and established cell-cell contacts, within 24 hours (Figure 1). The cardiomyocytes were observed to beat and to show electrical activity within 24 hours in culture. After 48 hours, the cultured cardiomyocytes displayed correlation of activity across individual electrode points of the MEA and Cx43 immunofluorescence (indicative of gap junctions) showing formation of an electrical syncytium. MEA recordings of electrical activity from these cryopreserved and fresh neonatal rat ventricular cardiomyocytes run in parallel show that the activity is time locked (synchronous) across all electrode locations (Figure 2). Spike activity and beating synchrony was maintained in long-term culture. The cryopreserved neonatal rat ventricular cardiomyocytes displayed electrical activity and responsiveness to drug treatment characteristic of freshly dissociated neonatal rat ventricular cardiomyocytes in culture (Figure 3). Treatment with isoproterenol or carbachol caused predictable and reversible alterations in excitation-contraction. In some experiments, 47-day cultures were treated with heptanol which inhibits gap-junction coupling (Figure 4). The effects on Cx43 immunofluorescence and the time of coupling events in the MEA were observed. Heptanol induced a significant increase in coupling time and this was reversed following drug washout.

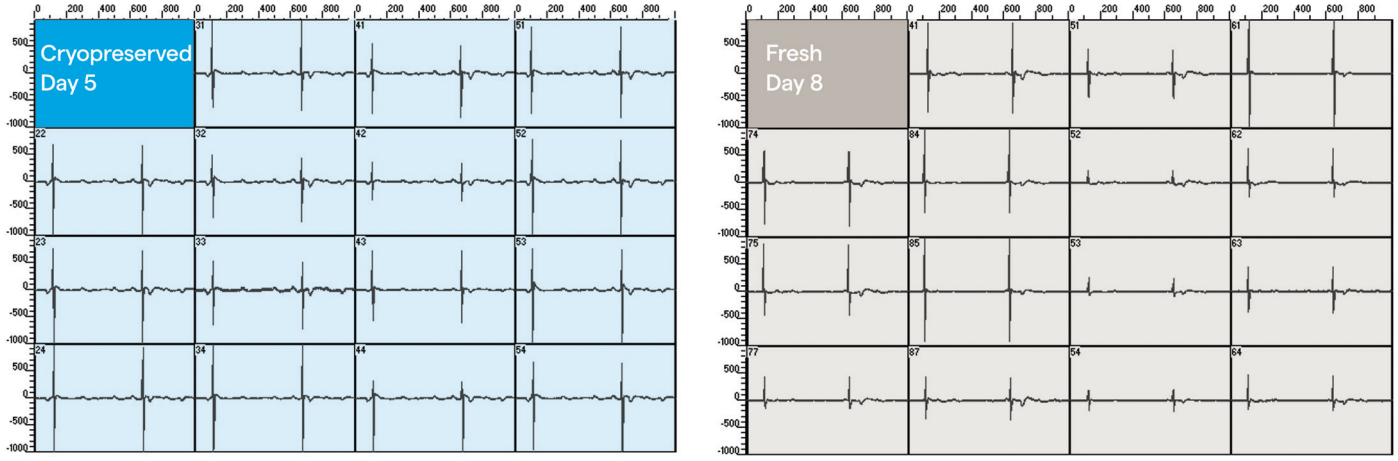

Figure 2
Cryopreserved cardiomyocytes retain electrical activity. MEA recordings of electrical activity from cryopreserved and fresh neonatal rat cardiomyocytes at 5 days and 8 days in culture respectively. In this example, the image represents a screen capture of the regular, spontaneous electrical activity taking place across 15 of 60 electrodes of the MEA array during a 1-second episode. The cardiomyocyte density on the cryopreserved sample electrode had an even distribution but in this instance the fresh culture was sparse over some electrode locations. A major point of note is that the activity is time locked (synchronous) across all electrode locations.

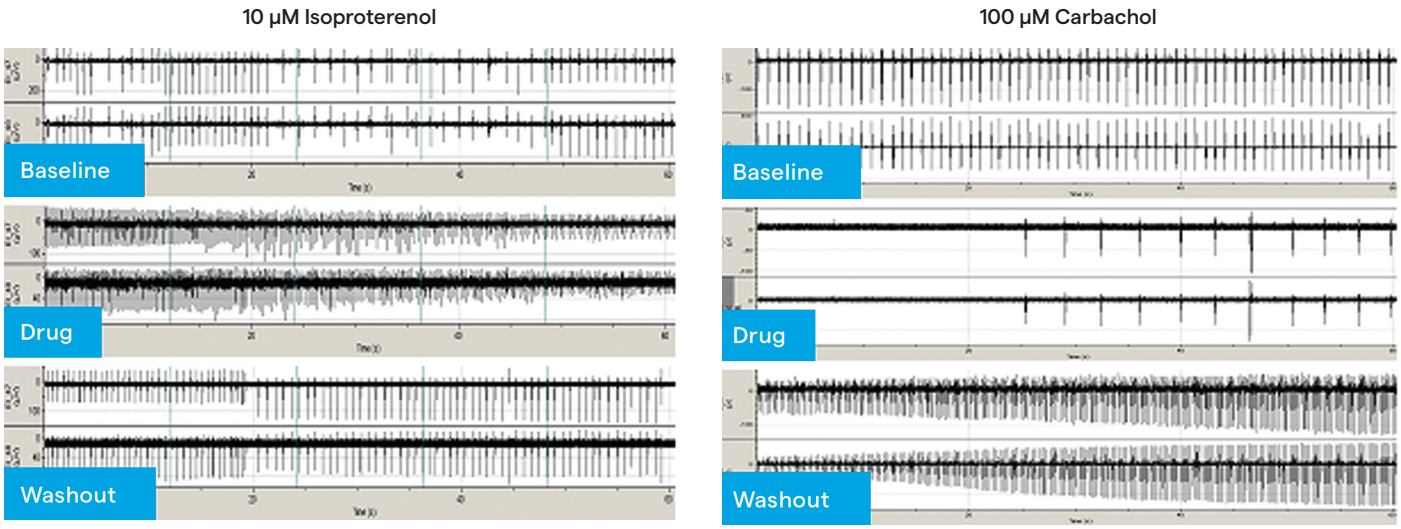

Figure 3.
Cardiomyocyte responses to cardioactive substances. Cardiomyocytes responded to 10 µM isoproterenol (β adrenergic agonist) and 100 µM carbachol (muscarinic cholinergic agonist). Washout reversed the acute drug effects observed.

Discussion

The cryopreserved cardiomyocytes displayed excellent morphology and viability in culture. Cardiomyocytes were spontaneously beating within 24 hours of plating and syncytial beating of the whole culture was evident by 48 hours. Gap-junction expression across all the cardiomyocytes and network electrical activity was well established at Day 7. We tested the cardiac cells in a MEA system for electrophysiological parameters associated with cell type, age, and effects of adrenergic and cholinergic drugs. The cryopreserved cardiomyocytes displayed robust activity across all MEA electrodes and acute exposure to the autonomic drugs gave predictable and reversible responses, comparable to freshly prepared cardiomyocytes (purified to the same level) in culture. Some of the cryopreserved cardiomyocyte cultures maintained on MEA were followed for up to 47 days and the network properties of the cardiac cells analyzed. Network signaling, which is reflected in coupling time between recording points, was robust for the cryopreserved cardiomyocyte cultures even at 47 days and network signaling could be dramatically (but reversibly) reduced by treatment with the gap-junction inhibitor heptanol.

Taken together, these data show that cryopreserved neonatal rat ventricular cardiomyocytes can be easily thawed and cultured to give *in vitro* cardiac cell test beds, ideal for a spectrum of assays. The cryopreserved cardiac cells display excellent viability, morphology and connectivity as well

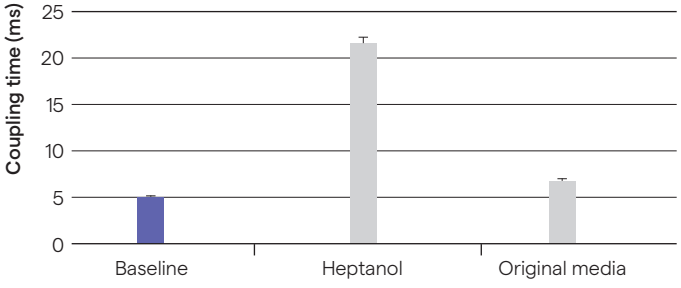

Figure 4.
Change in signal propagation upon heptanol treatment. Cryopreserved cardiomyocytes were thawed and cultured 47 days. Treatment with 0.5 mM heptanol (reversible gap-junction inhibitor) increased coupling times between cells (i.e. between electrodes). Washout of drug restored the original untreated coupling time. Matrix analysis confirmed that the heptanol-induced increase in coupling time was observed throughout the MEA.

as early establishment of contractile and electrical activity necessary for use in a functional screening/pharmacology assay. They provide the researcher with a ready-to-use, defined cardiac cell test bed for the long-term evaluation of toxins and drug candidates. For the laboratory where animal handling/dissection and cardiac tissue preparation are difficult or else problematic, the availability of high quality, batch tested, viable neonatal rat ventricular cardiomyocytes which can be simply thawed and cultured, represents new research and development opportunities. For the cardiac drug discovery/screening laboratories requiring high quality, and batch to batch reliability guaranteed, these easy-to-use cryopreserved ventricular cardiomyocytes represent significant time savings for R&D programs.

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