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Recommendations for Short Term Culturing of Viable Rod Shaped Rat Cardiomyocytes

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Abstract

In vitro primary cultures of isolated adult rat cardiomyocytes are becoming an increasingly popular model to study heart muscle stressors. This model can easily be manipulated in a controlled environment and results obtained can provide valuable insights into the pathophysiology of heart disease. Over the past years several improved methodologies have been described in the development of a robust technique to help maintain cardiomyocytes in culture. However, despite these advances, culturing of primary cardiomyocytes remains a challenge. In this study, we present a simple yet reproducible method for isolation and culture of viable rod shaped cardiomyocytes. Cardiomyocytes were maintained in supplemented Media 199 with or without foetal bovine serum. Their viability was assessed using trypan blue while the metabolic activity was measured using an Adenosine 5'-triphosphate assay. Results obtained in this study were used to provide general guidelines to evade pitfalls related to low cardiomyocyte yields and subsequent poor culturability of cardiomyocytes. Isolated cardiomyocytes cultured in the presence of foetal bovine serum maintained their *in vitro* striated rod shaped morphology for 72 hours in culture, after which, they flattened and spread out. Whereas, cardiomyocytes cultured in the absence of foetal bovine serum remained rod shaped for up to 120 hours.

Keywords: Adult rat cardiomyocytes; Culture; Foetal bovine serum; Laminin coated plates; Rat heart

Introduction

The heart is an essential organ that plays a pivotal role in the transport of oxygenated blood and essential nutrients through the body. During myocardial damage, the myocardium does not regenerate, as adult cardiomyocytes are terminally differentiated. Damage to the cardiomyocytes *per se* forms the basis of several cardiovascular related disorders. Heart failure is usually as a result of arterial blockage that prevents oxygenated blood from flowing to the heart muscle. During acute oxygen deprivation, myocardial tissue undergoes necrosis and this result in a non-contractile fibrotic scar. To compensate for diminished contractibility, the remaining cardiac muscle undergoes hypertrophy [1].

More than 30 years ago a rapid method for the isolation of adult rat cardiomyocytes was described for the first time [2]. To date, cardiomyocytes have been isolated from various animals including mice, rats, dogs, rabbits and guinea pigs [3-11]. Isolation of cardiomyocytes from the above mentioned model organisms can provide researchers with a tool to study morphological changes that occur in the middle of a heart muscle [10]. In the heart, cardiomyocytes are strongly connected to each other by junctional complexes or intercalating discs making isolation of single rod shaped cardiomyocytes difficult [10]. Additionally, culturing of these rod shape cardiomyocytes is challenging as cardiomyocytes are known to change morphology in a process termed "cardiomyocyte dedifferentiation" and this remains an obstacle to most pathophysiological studies [12].

Jacobson and Piper [13] described two methods for culturing cardiomyocytes. The one method is to culture cardiomyocytes in a serum supplemented medium but without extracellular attachment matrix like laminin or collagenase. After 2 days in this media, cardiomyocytes attach and start to "spread", sending out pseudopodia and losing their rod shaped morphology. The second method includes the use of serum free media with an attachment matrix. With this method it is proposed that cardiomyocytes adhere to the matrix within 3 hours retaining their rod shaped and striated appearance. Although optimal isolation of primary cardiomyocytes may be achieved, successful culture of rod shaped viable cardiomyocytes remains a big challenge [1,14]. Thus, the aim of this study was to provide recommendations on an in house reproducible method used to isolate and culture large numbers of viable rod shaped cardiomyocytes in the presence and absence of Foetal bovine serum (FBS).

Materials and Methods

Reagents

Unless specified all chemicals were of an analytical grade and obtained from Sigma Aldrich (St. Louis, MO, USA). Foetal Bovine Serum (FBS), Dulbecco's Phosphate Buffered Saline (DPBS), Media 199, penicillin streptomycin amphoterin B (pen/strep/ampB) were bought from LonzaBioWhittaker® (Walkersville, MD, USA). Collagenase type II was procured from Worthington Biochemical Corporation (Lakewood, NJ, USA). Fatty acid free BSA was bought from Roche (South San Francis, CA, and USA). Trypan blue dye was obtained from Invitrogen (Carlsbad, CA, USA) and taurine was obtained from Acros Organics (Geel, Belgium).

Cardiomyocyte isolation

Four month old male Wistar rats weighing in the range of 350-400 g were used in this study. All procedures were performed in accordance with the MRC guidelines for care and use of laboratory animals (http://www.mrc.ac.za/ethics/ethicsbook3.pdf).Wistar rats were anaesthetised with sodium pentobarbital (40 mg/kg body weight) by a single intraperitoneal injection. Hearts were removed and rapidly rinsed in calcium free ice cold Kreb'sHenseleit buffer (prepared in dH_2O containing: 119 mMNaCl, 24.9 mM$NaHCO_3$, 4.74 mMKCl, 1.19 mM KH_2PO_4, 0.6 mM$MgSO_4$, 0.59 mM$NaSO_4$, and 11 mM glucose, pH 7.4) and transferred to a Langendorff perfusion apparatus. The aorta was fixed to a cannula and perfusion was initiated. Pressure was kept above 60 kPa whilst perfusion solutions were continuously gassed with medical oxygen. Temperature of the perfusion liquid was kept constant at 37°C using a circulating water bath. To wash out residual blood, the hearts were perfused with solution A (prepared in dH_2O containing: 6 mMKCl, 1 mM Na_2HPO_4, 0.2 mMNaH_2PO_4, 1.4 $MgSO_4$, 128 mMNaCl, 5.5 mM glucose, 10 mM HEPES, pH 7.4) for 5 minutes. After 5 minutes, the buffer was switched to solution B. Hearts were digested with 75 ml of solution B (containing: 2 mM glutamic acid, 0.1% (w/v) collagenase type II, 5 mM 2,3 Butanedionemonoxime, 2 mMcarnitine, 5 mMtaurine and 100 mM$CaCl_2$ dissolved in "solution A") for a total of 35 minutes at 37°C. During that period the calcium (Ca^{2+}) concentration of solution B was increased gradually; by the addition of 37 µl and 50 µl of a 100 mM$CaCl_2$ after 10 and 15 minutes, respectively. The enzymatic digestion was continued for a further 10-15 minutes. The digestion was complete when the solution flowed freely (instead of dripping) from the heart. The digested heart was removed from the Langendorff apparatus, the atria cut off and the heart ventricles minced into small pieces using sterile scissors. The minced heart tissue was then submersed into oxygenated solution C (dissolved in 25 ml of solution A + 25 ml of solution B" containing: 1% BSA (fatty acid free), 1% BSA and 100 mM$CaCl_2$) and then placed in a shaking water bath (75 strokes/minutes) at 37°C for 15 minutes. The Ca^{2+} concentration during incubation was raised again by adding 100 µl of 100 mM$CaCl_2$ after 16, 17, 18, 19 and 20 minutes respectively, to obtain a final concentration of 1.25 mM$CaCl_2$. At each of these time points the cells were gently triturated with a 10 ml serological pipette. The myocytes were filtered through a 200 µm pore size nylon mesh and gently spun down at 30 x g for 3 minutes at room temperature (RT). The supernatant was aspirated and the pellet of cells was gently resuspended (not to disturb the pellet) by overlaying in 25 ml of solution D (dissolved in "solution A" containing: 2% BSA (fatty acid free) and 100 mM$CaCl_2$) at RT. After 5 minutes, solution D was aspirated and the cell pellet was resuspended in 10 ml of supplemented medium 199 (5 mMcarnitine, 5 mMtaurine, 0,1 mMBrdU, 5 mMcreatine monohydrate, 5% FBS and 0.5% pen/strep/ ampB). Before continuation of the experiment, cell viability was determined by trypan blue exclusion assay and a cell viability ≥70% was required for subsequent culturing experiments.

Culturing of cardiomyocytes

To remove debris and non-myocytic cells, the isolated cells were pre-plated in a 60 mm Petri dish for 1 hour under standard tissue culture conditions (CO_2 incubator with 5% CO_2 in air at 37°C with relative humidity >80%). After 1 hour of incubation, the non-adherent myocytic cells (cardiomyocytes) were removed by aspiration and transferred to a sterile 50 ml tube and cell viability count was repeated to confirm ≥70% cell viability. Cells were plated at a seeding density of 5.94 x 10^5/3 ml on laminin coated 6 well plates (prepared according to the manufacturer's instructions) and incubated under standard tissue culture conditions for 4 hours in supplemented Media 199 (5 mMcarnitine, 5 mMtaurine, 0.1 mM 5-Bromo-2´-Deoxyuridine (BrdU), 5 mMcreatine monohydrate, 5% FBS and 0.5 pen/strep). After 4 hours the media was aspirated and the cardiomyocytes cultured overnight in fresh supplemented Media 199 but without FBS. The following day (24 hours after isolation), a long term culture was setup by incubating cardiomyocytes in Media 199 (5 mMcarnitine, 5 mMtaurine and 5 mMcreatine monohydrate) with or without 5% FBS under standard tissue culture conditions. Media was refreshed every second day. Metabolic activity (ATP assay) and cell viability (trypan blue assay) were assessed daily until cardiomyocyte dedifferentiation occurred. During this period cardiomyocyte contractions were measured by randomly focusing on one cardiomyocyte at a time and recording the contractions for 1 minute using Nikon Eclipse Ti inverted microscope (Nikon, Tokyo, Japan).

Cell viability assessment

The trypan blue exclusion assay was used to determine cell viability according to manufacturer's instructions. Viable (unstained) and non-viable (stained) cells were counted using Nikon Eclipse Ti inverted microscope. For statistical analysis, 5 non overlapping fields using an x20 objective were taken for each individual well.

Metabolic activity (ATP assay)

Intracellular ATP concentrations were determined using a ViaLight™ plus kit (Lonza, Basel, Switzerland), following manufacturer's instructions. Luminescence was measured using a BioTek FLx800 plate reader (BioTek Instruments Inc., Winooski, VT, USA).

Results and Discussion

In this study, a reproducible protocol for culturing rod shaped cardiomyocytes was investigated and several critical factors to improve cardiomyocyte viability and yield were identified. Failure to adhere to these factors resulted in a loss of cell viability with a cardiomyocytes yield of ~40% (data not shown). Despite the fact that fibroblasts, endothelial cells and smooth muscle cells make up 60-70% of heart cells [15] the modified protocol consistently yielded ~4 x10^6 rod shaped cardiomyocytes per heart. Using this protocol we were able to culture rod shaped cardiomyocytes for several days under different conditions (Figure 1A and Figure 1B).

Isolation of striated rod shape beating cardiomyocytes forms the basis of myocytic cell survival in culture. The following recommendations on the isolation of cardiomyocytesproved to be critical for subsequent culturing. (a) Minimise delays between heart excision and perfusion. (b) In addition, cannulating and ligation of the aorta should allow for adequate movement of the heart. Inserting the cannula to deeply into the heart will result in insufficient perfusion and inadequate digestion of the heart. (c) Proper enzyme selection is critical for successful isolation. A new batch of collagenase should be tested as batch variation can affect digestion and cell viability. (d) Calcium should be added gradually to prevent a sudden Ca^{2+} overload and thus the isolation of contracted cardiomyocytes. (e) Sufficient trituration of the isolated cells is needed to liberate single cardiomyocytes and improve yields. (f) Finally, after isolation, pre-

plating of cardiomyocytes for a short period is essential to separate the non-adherent cardiomyocytes from other adherent cells.

Next, we defined factors to sustain cardiomyocytes in culture. The initial viability of rod shaped cardiomyocytes after isolation should be as high as possible (not less than 70%). Laminin, fibronectin, collagen and gelatin are some of the known cell attachment methods [16,17]. According to Bird [18], laminin is the preferred cell attachment matrix for culturing of adult rat cardiomyocytes. In this study, we observed that laminin provided an enhanced cell attachment matrix. The concentration of laminin used is of importance. Laminin provide better cell attachment and survival if used at ≥ 25 µg/ml. Cardiomyocytes were attached within 4 hours of initial plating exhibiting spontaneous rhythmic contractions, as visualised using a light microscope (data not shown). To inhibit actively proliferating interstitial cells, still present after pre-platting, the isolated cardiomyocytes were co-cultured with BrdU. Plating of cardiomyocytes at the correct density to ensure a monolayer is critical for cell attachment prior to the first wash. Additionally, cultured cardiomyocytes are sensitive to medium turbulence. Thus, care should be taken during media changes.

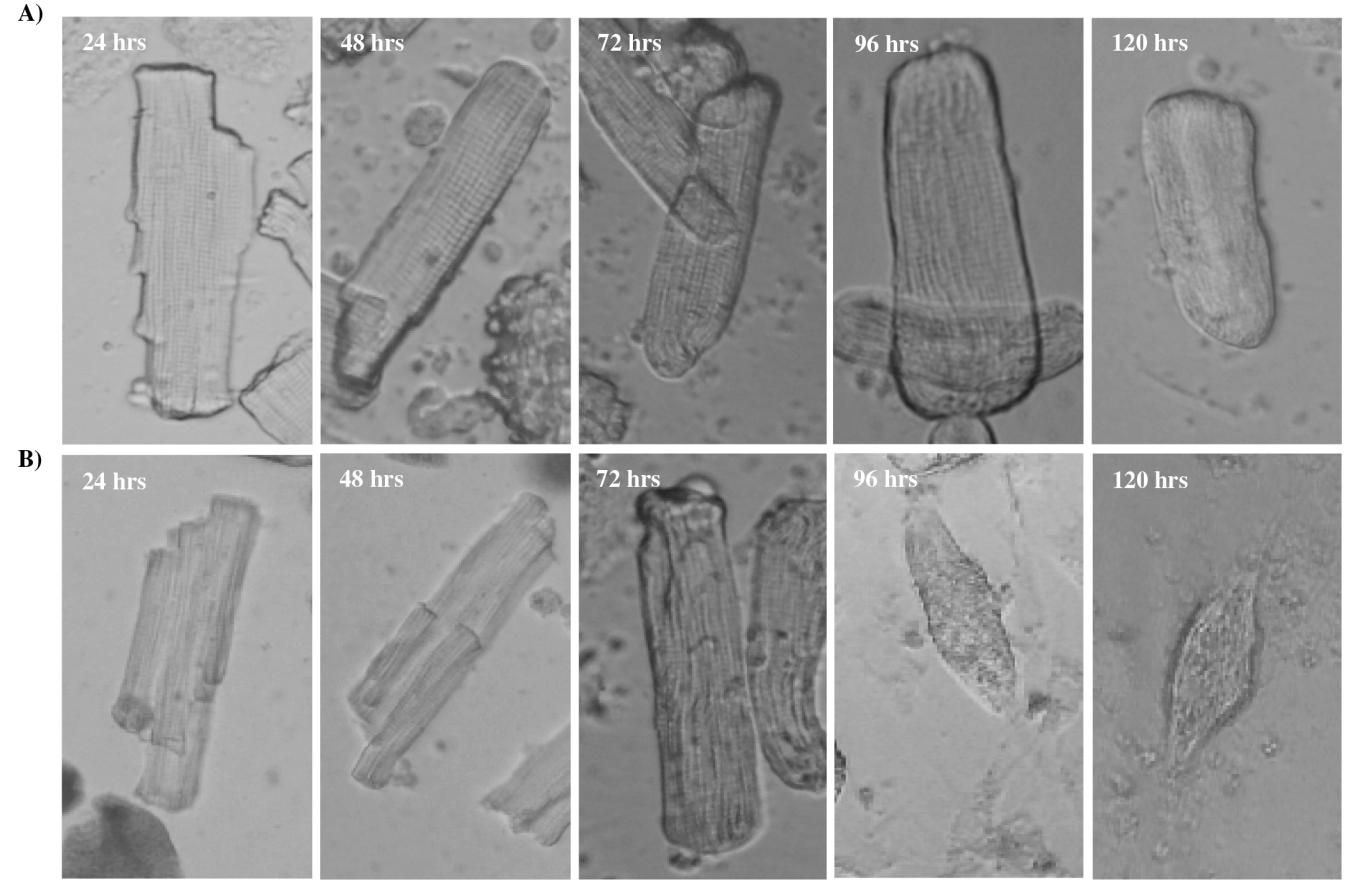

Figure 1:Representative images of phase contrast micrographs of isolated adult rat cardiac myocytes. Panel **(A)** depicts cardiomyocytes cultured in serum free medium. Panel **(B)** represents cardiomyocytes cultured in 5% FBS medium. (24 hours) Denotes 24 hours in culture, (48 hours) 48 hours in culture, (72 hours) 72 hours in culture, (96 hours) 96 hours in culture and (120 hours) 120 hours in culture. Images were obtained from x20 magnification respectively.

Foetal bovine serum plays an integral role in the survival and morphology of cardiomyocytes. In this study we used Media 199 supplemented with or without FBS. Foetal bovine serum is a rich nutrient source. The supplementation of medium with 5-15% FBS provides additional hormones and growth factors that enhance the survival of cells for an extended period of time [14,18,19]. Subsequently, we tested our culturing method in the presence and absence FBS. After overnight culture, cells were refreshed with supplemented Media 199 with and without the addition of 5% FBS. A cell viability of 70% ± 12% was obtained after 24 hours in culture; of which 80% ± 4% were rod shaped irrespective of culture medium (Figure 2B and Figure 3B). Furthermore, spontaneous rhythmic contractions were observed under both culture conditions implying that FBS had no effect on the morphology or contractibility of cardiomyocytes after 24 hours in culture. Similar results were observed for cells cultured for 48 hours. At 72 hours in culture, a decrease in rod shaped morphology was observed for cells cultured with or without FBS (52% ± 11% and 60% ±10% with a cell viability of 59% ± 6% and 53% ± 7%, respectively) (Figure 2 and Figure 3). This result correlated with results obtained for ATP activity (>65% irrespective of culture media). As expected, cells cultured in the presence of FBS for 96-120 hours lost their rod shaped phenotype (Figure 2B and Figure 1B). In contrast, cardiomyocytes cultured without FBS retained their rod shaped morphology for up to 5 days albeit at the expense of cell

viability (27% ± 9% compared to 6% ± 4% and 57% ± 9% compared to 37% ± 5%) (Figure 2A and Figure 3A). Similar results were reported by other studies [11,14]. However, while serum free medium retain rod shape morphology, ATP as a measurement of metabolic activity and intracellular energy levels were found to be dramatically decreased in cell cultured in serum free medium over time. Although not investigated in this study, decreased ATP production in cultured cardiomyocytes has been associated with impaired mitochondrial bioenergetics capacity and decreased contractibility due to the rapture of the sarcoplasmic reticulum and subsequent disruption of Ca^{2+} homeostasis. Furthermore, Mitcheson et al. [14] reported that cells cultured in serum free medium for a prolonged period of time showed an atrophic phenotype with a decrease in protein express even after supplementation with creatine and L-carnitine. In terms of contractility, FBS appeared to have a modulating effect. Cardiomyocytes cultured with or without of FBS showed similar beating activity with a beating frequency of >35 beats per minute (up to 72 hours in culture). After 72 hours, a reduced beating activity (>20 beats per minute) was observed with cardiomyocytes cultured in serum free medium having a slightly reduced beating activity compared to cardiomyocytes cultured in FBS.

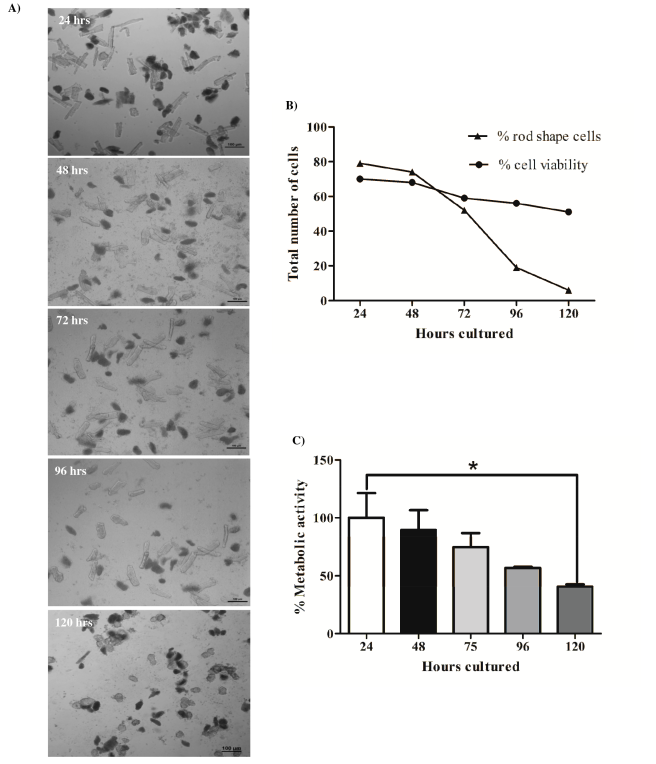

Figure 2: Representative images of isolated primary cardiomyocytes cultured in media supplemented with 5% FBS for 120 hours. Panel **(A)** depicts cardiomyocytes cultured for 120 hours (24 hours-120 hours) and stained with trypan blue dye, **(B)** shows trypan blue stained cells and rod shape morphology of cardiomyocytes, **(C)** ATP metabolic activity of cardiomyocytes in culture. The results are the mean ± SEM of n=3 independent experiments. A p value < 0.05 was deemed as significant. *Represents a statistical significance when compared to 24 hours of culture.

Culturing of primary rat cardiomyocytes has become an intriguing model to study cardiomyocytes*in vitro*. The isolation and culture of sufficient numbers of contracting rod shape cardiomyocytes is feasible but remains challenging. The method described in this study allows for the reproducible isolation of large numbers of cardiomyocytes from a single heart and subsequent culture for an extended period of time. Depending on the downstream application, the authors suggest that cardiomyocytes should be cultured in serum free medium for a short period of time or researchers should investigate the use of gravity less culture methods such as three dimensional culturing systems for longer term culturing of isolated adult rat cardiomyocytes.

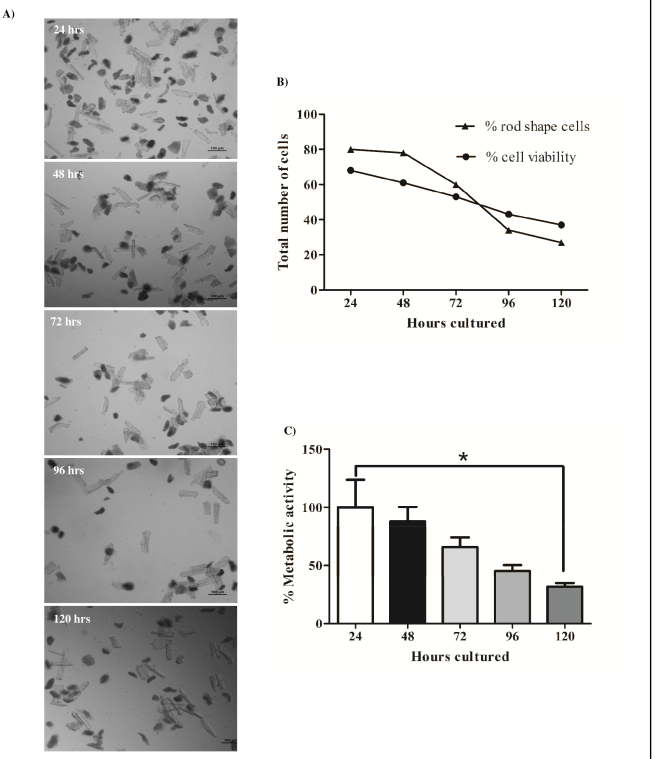

Figure 3:Representative images of cardiomyocytes cultured in serum free medium for 120 hours. Panel **A)** depicts cardiomyocytes cultured for 120 hours (24 hours-120 hours) and stained with trypan blue dye, **(B)** shows trypan blue stained cells and rod shape morphology of cardiomyocytes, **(C)** ATP metabolic activity of cardiomyocytes in culture. The results are the mean ± SEM n=3 independent experiments. A p value < 0.05 was deemed as significant. *Represents a statistical significance when compared to 24 hours of culture.

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