An optimized protocol for culture of cardiomyocyte from neonatal rat

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Abstract

Primary culture of cardiomyocytes has been widely used as a valuable tool for pharmacological and toxicological studies. However, the fact that heart is a solid organ and cardiomyocytes do not proliferate after birth makes the primary myocardial culture a tedious job. The present study reports an improved method for rapid isolation of cardiomyocytes, as well as the culture maintenance and quality assurance. The whole culture process can be shortened to 3.5 h by reducing enzyme digestion period. Moreover, the new protocol guarantees cell yield and viability, and produces more than 95% cardiomyocytes in culture. The cardiomyocytes can respond to Angiotension II stimulation with increased protein synthesis, suggesting the practical value of this new culture method.

Introduction

Since Harary and Farley first separated Wistar neonatal rat cardiomyocytes and succeeded in making the myocardiocytes exhibit spontaneously beating activity for 40 days *in vitro* (DIV) (Harary and Farley 1960), primary culture of cardiomyocyte has been widely applied in the basic cardiological research. One advantage in doing experiments with neonatal rat cardiomyocytes is lack of the influences of hemodynamic factors existing *in vivo*. In addition, in cell culture it is feasible to control other concomitant factors artificially.

The culture method of neonatal rat cardiomyocytes established by Harary and Farley has been modified by many scientists in the past 40 years (Harary and Farley 1963). Two isolation steps are essential for a successful culture. One is the enzyme digestion step for dissociating cells

from heart tissue. The other is the purification step for eliminating non-muscle cells. The latter is critical for ensuring a constant proportion of myocytes. Several techniques have been applied to reach this goal, including differential attachment technique (Blondel et al. 1971), deprivation of serum (Shields et al. 1988), density gradient centrifugation (Flanders et al. 1995; Harada et al. 1998), and using chemical reagents to inhibit non-muscle cells proliferation (Simpson and Savion 1982). To make a good culture with high myocytes yield, repetitive trypsinization of heart tissue in short periods is often recommended, which generates a higher proportion of undamaged muscle cells, other than a single digestion for longer time (Mark and Strasser 1966). Normally, the incubation time with trypsin depends on the amount of undigested tissue. Although repetitive digestion can give good yield, it could also lead to poor cell viability when the minced tissue is exposed to

enzyme solution for too long. That is because the heart is one of the most delicate organs sensitive to environmental changes. Therefore, the isolation and culture of neonatal cardiomyocytes require experience and skills for ensuring good yield and high quality cell culture. Moreover, because cardiomyocytes lose their ability to proliferate shortly after birth, growth of heart tissue is governed by cell growth rather than by proliferation. This feature of cardiomyocytes does not allow cell propagation and requires repetitive primary cultures for multiple experiments. Hence, the application of cardiomyocyte culture is somehow limited because of the heavy preparation duty.

The present study reports a convenient, reliable and time-saving protocol for making consistent cultures of high-yield and high-quality cardiomyocytes. By decreasing the digestion periods, the whole culture process only takes 3.5 h, including a 1.5-h purification step. The yield is 3×10^6 cells from one animal with 90% viable cells, which is comparable to the conventional culture protocol (Paul Simpson 1985)

Materials

Equipments

Magnetic stirrer (with heating function) (IKA¹, RH-T/C)

Liquid scintillation counter (Beckman², LS 6000)

Solutions/supplies

Culture medium: One packet of DMEM powder (GIBCO³, 12800-017) is dissolved in 1000 ml of distilled water, 2.2 g sodium bicarbonate and 25 mM Hydroxyethyl piperazine ethanesulfonic acid (HEPES) (Sigma⁴, H4034) are added and adjust the pH value to 7.3. This solution is sterilized by filter-sterilization. The following sterile solutions are added to complete the DMEM: 10% (v/v) heat-inactivated fetal bovine serum (Hang

Zhou Sijiqing⁵, 050416), Antibiotics stock mixture (Hyclone⁶, SV30010).

Enzyme solution: 0.08% trypsin solution is freshly prepared to ensure the enzyme activity. Dissolve trypsin (Sigma⁴, T8003) in Ca^{2+} and Mg^{2+} -free PBS buffer either at room temperature for 4 h with agitation or at 4 °C overnight. Sterilize the solution by filtration through a 0.22 μm filter.

PBS solution: one packet of Ca^{2+} and Mg^{2+} -free PBS powder (Boster⁷, AR0030) is dissolved in 2 l dH_2O . The pH value is adjusted to 7.3. The solution is sterilized either by autoclaving or filter-sterilization.

Deoxyribonuclease I (sigma⁴, 31135) is dissolved in sterile PBS buffer to make a stock solution of 5 mg ml^{-1} , dilute the solution to 50 $\mu\text{g ml}^{-1}$ with trypsin solution before use.

5-Bromo-2'-deoxyuridine (BrdU) (Aldrich⁴, 858811) is dissolved in sterile dH_2O to a stock solution of 100 mmol l^{-1} , final concentration is 0.1 mmol l^{-1} .

Angiotensin II (Sigma⁴, A9525) is dissolved in sterile dH_2O to a stock solution of 10^{-3} mol l^{-1} . Dilute it to appropriate concentration with serum free medium before use.

Cell culture plates (diameter 35 mm) and flasks (Greiner bio-one⁸, 657 160, 690 170 respectively).

Anti- α -sarcomeric actin ((Sigma⁴, A2172) is diluted to 1:600 with dH_2O before use.

SABC immunoenzymatic staining kit (Boster⁷, AR0030)

Procedures

Preparation of cardiomyocyte culture

1. Anesthetization and sterilization: Rat pups (Sprague-Dawley or Wistar rats) at the age of postnatal day 1–3 were sacrificed by ethyl ether. The animals were decontaminated with 75% ethanol, and transferred to a Luminer flow hood.

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2. Dissection (performed on ice): Surgically remove the beating heart from animals immediately, and keep it in cold Ca^{2+} and Mg^{2+} -free PBS buffer. Ventricles were excised and transferred to fresh ice-cold PBS buffer and were minced with fine scissors into $1\text{--}3\text{ mm}^3$ pieces after washing blood away from the heart lumen. Red blood cells were removed by instant centrifugation for two times.

3. Preconditioning (20 min): A preconditioning step was introduced in the present protocol prior to trypsinization. The minced tissue was transferred to a 40 ml conical flask containing trypsin solution (0.08%, 0.5 ml per rat) and a small magnetic bead. The flask was then settled on ice for 20 min, and shaken every 3 min for better mixing.

4. Trypsinization (10 min): Following preconditioning, the tissue was digested in the conical flask at 37°C for 10 min, which was subjected to constant stirring (150–200 rpm).

5. Centrifugation (5 min): After trypsinization, cells were dispersed from the tissue by gently pipetting. The cell suspension was settled on ice for several seconds. The supernatant was carefully transferred to a 15 ml centrifuge tube. Trypsin activity was inhibited by adding a mixture of trypsin inhibitor and cold culture medium supplemented with 10% FBS (1:1, v/v). Cell pellet was formed by spinning at 1000 rpm for 5 min, and was resuspended in 2 ml warm culture medium.

6. Repeating trypsinization: The remaining tissue in the conical flask from step 5 was continuously digested by adding 5–10 ml fresh pre-warmed trypsin solution containing DNase I (0.05 mg ml^{-1}). Depending on the amount of undigested tissue, trypsinization and centrifugation steps were repeated for 2 to 3 times (25–35 min).

7. Cell harvest (10 min): Cell suspension from all dissociated steps was pooled in one centrifuge tube and settled for 5 min. The suspension was gently transferred to a new centrifuge tube excluding the precipitates on the bottom. Cells were harvested by centrifugation for 5 min at 1000 rpm. Finally the cells were plated in a 40 ml tissue culture flask and incubated at 37°C in a humidified atmosphere (5% CO_2 , 95% air).

8. Purification (1.5 h): Myocardocytes enriched culture is obtained through the following two steps. Since non-myocardocytes attach to the substrata more readily than myocardocytes,

firstly, cells harvested from step 7 were incubated for 1.5 h to allow the attachment of non-myocardocytes. The majority of myocardocytes remained in culture medium. The suspended cells were collected and plated at a density of $2 \times 10^5\text{ ml}^{-1}$ into a new tissue culture flask. BrdU (0.1 mmol l^{-1}) was then added to the culture medium for 48 h to prevent proliferation of non-myocardocytes that might be present in the culture.

9. Cultivation: Generally, cells isolated from 2 to 3 hearts can be seeded in one 40 ml culture flask. The cells should not be disturbed during the initial 24 h. The culture medium was replaced with fresh media without BrdU for every 48 h.

Cell yield and viability evaluation

The yield and viability of the culture was monitored by dye exclusion using trypan blue (0.4%). Mix 1 drop of trypan blue (0.4%) with 9 drops of cell suspension and allow 1–2 min for absorption. Cells excluding the staining are considered viable and the percentage of non-blue cells is used as an index of viability. Count both the total number of cells and the number of stained (dark) cells by a hemocytometer for measuring the yield and viability as follows:

$$\text{Yield} = (\text{total number of cells in four grids}/4) \times 10^4 \times (\text{cell suspension volume})$$

$$\text{Viability}(\%) = (\text{Total cells counted} - \text{stained cells})/\text{total cells counted} \times 100$$

Immunoenzymatic staining assay

Since α -sarcomeric actin is considered as a specific protein in cardiomyocytes, a mouse monoclonal anti- α -sarcomeric actin (1:600, Sigma) was applied as the primary antibody to identify cardiomyocytes in the culture. A goat anti-mouse biotinylated immunoglobulin conjugated with avidin-biotinylated horseradish peroxidase was used as the secondary antibody followed by ABC

staining systems according to the manufacturer's instructions with minor modifications. Cardiac fibroblasts were also stained as a negative control.\

³[H]-leucine incorporation assay

Cells were rinsed twice with ice-cold PBS and then incubated in 5% trichloroacetic acid at 4 °C for 1 h. Protein precipitates were washed twice with ice-cold water and dissolved in 1 ml 100 mmol l⁻¹ NaOH. The radioactivity was determined by a liquid scintillation counter.

Statistical analysis

All values in the text and figures are presented as mean ± S.E. of (*n*) independent experiments. All data were analyzed by one-way ANOVA followed by Tukey test. *p* values ≤ 0.05 were considered statistically significant.

Results and discussion

Primary culture of cardiomyocytes has been widely used as a valuable tool for pharmacological and toxicological studies. Based on the established cardiomyocyte culture methods, scientists have made significant progress in basic cardiological research including exploration of the molecular mechanism, management and prevention of heart diseases. Therefore, this type of cell model has great potential for studying the cellular and molecular aspects of cardiac alterations under injury.

Despite the success achieved based on the established culture methods, there are still major problems that need to be overcome. The fact that cardiomyocytes lose their ability to proliferate shortly after birth makes the primary culture a repetitive job. In addition, the fact that heart tissue needs more time for dissociation makes the culture process very lengthy. As suggested by Mark and Strasser (1966), repeated short periods of incubation with trypsin are often used than a single digestion for longer time. The more repeated times, the more isolated cells you can get, however, over-digestion is easily caused which might be one of the main reasons of non-attachment or weak contraction of cardiomyocytes. On the contrary,

fewer cycles and/or shorter duration may lead to low yield but ensure high-quality cardiomyocytes. According to Chlopelkova et al. (2001) and Gorelik et al. (2004), five to eight incubations with trypsin (10–20 min for each cycle) were mostly applied in the literature. Given the variations between individuals, it is hard to determine an explicit and uniform time for the whole process. Typically, at least 100 min are required for the entire digestion process (Orlowski and Lingrel 1990). Thus, a new method that enables fast isolation and cultivation of cardiomyocytes will make this cell model more applicable. To this end, we developed an improved culture protocol for fast isolation and stable cultivation of cardiomyocytes.

In the present study, we introduced an optimized protocol for cardiomyocytes culture, which is rapid, convenient and labor saving. Our protocol is superior to other available culture methods because the digestion period has been greatly reduced to 30–40 min (for 3–4 repetitive digestions). Following the present protocol, the whole dissociation process takes only about 3.5 h, including a 1.5 h purification step. However, as we show below, the yield, viability, and conditions of the culture are still comparable to what has been reported.

It is normally anticipated that an optimized culture protocol would give both good yield and high cell viability. In our experiment, these two indicators were analyzed by trypan blue exclusion assay, and subsequently, cell counts with a hemocytometer. These assays were performed right after the 1.5 h purification step. As a result, about 3×10^6 myocytes were obtained from one heart in our protocol, which is almost the same compared to the conventional protocol (Simpson 1985). To further compare the yield from the optimized protocol and the classical one, we counted isolated cells from every trypsinization cycle while performing the two respective methods. As shown in Figure 1, the optimized protocol yielded about 8.4×10^6 isolated cells from one rat after four cycles of trypsinization, however, using the conventional protocol only repeated trypsinization steps (up to seven rounds) could lead to the same yield. Although dye exclusion assay may somehow overestimate cell viability, it is still a valuable tool for a quick determination of cell dissociation performance during isolation. It is very encouraging that 90% cells were still alive following our

Figure 1. Comparison of the yield of isolated cells obtained by using the optimized and the classical protocol. Data shown are the means of quintuplicate determinations $\pm$ S.E. The figure represents three experiments.

culture procedures. The cell viability was further examined by tracking the contraction rate of cardiomyocytes for 20 days in culture, where a constant beating activity could still be observed.

In our culture, attachment of cardiomyocytes occurred within the first day. These cells also began to spread out to have their morphological differentiation on day 1 in culture. On the second day, the myocardiocytes looked elongated. Most of them were uninucleate while some were binucleate. On 3 DIV, a confluent monolayer culture was observed when cardiomyocytes started to cross with each other by growing their pseudopodia (Figure 2). Some of the cardiomyocytes exhibited spontaneously beating activity from day 1 after attachment. Most of the cardiomyocytes beat at the same frequency when a monolayer was formed on the third day. The average beating rate was 150 beats per minute, which remained till day 20. Figure 3 showed the average beating rate of cardiomyocytes from 5 random fields on day 3, 10, 20 respectively.

The purity of our cardiomyocyte culture was determined by immunocytochemistry with an anti- α -sarcomeric actin mouse antibody, a well-known protein marker in the cytosol of cardiomyocytes. As shown in Figure 4, the immunoreactivity was observed in more than 95% cells on 3 DIV in our culture. Cardiac fibroblasts were devoid of staining, indicating that the primary antibody is specific for cardiomyocytes (Figure 5). These results demonstrate that cardiomyocytes represent major cell type in our culture.

All experiments above have been repeated for at least 3 times. Thus, we are able to conclude that our improved protocol provides an efficient way

for making a good cardiomyocyte culture. The significance of our method exists in the shortened period of culture procedures, which makes this cell model more practicable to researchers. As we stated above, the digestion time has been greatly reduced in our protocol (10 min for 3–4 times). This is mainly due to the new preconditioning step, i.e. 20 min incubation with trypsin on ice prior to digestion at 37 °C, which boosts the efficiency of trypsin. This method has been previously reported by Laue et al. (1995) for isolating islet cells. To preserve the tissue and to enhance the yield of cells, they used a preconditioning step at 4 °C permitting a maximal diffusion of the enzyme into the tissue to obtain equal enzyme activities throughout the tissue. Similarly, Landes et al. (2001) performed cold pre-incubation using collagenase in order to get a parathyroid cell culture with improved yield and functionality. It is noteworthy that this cold pre-incubation step ensures, and possibly even enhances the cell yield and viability of the culture. It is common that dissociation of cells from solid organs often induces a functional impairment due to the proteolytic cell damage by the applied digestive enzyme like collagenase, trypsin or dispase. Incubation with enzyme at low temperature alone can greatly diminish the entry of enzyme into the cells which causes cell damage. Therefore, this method was adopted and modified by us for the improved cardiomyocyte culture protocol. In the present study, a complete diffusion of trypsin into the minced tissue was obtained by cold pre-incubation prior to trypsinization, hence subsequent digestions were facilitated by ensuring that trypsin completely interacts with minced tissue when later

Figure 2. Cultured cardiomyocytes on 3 DIV. Cardiomyocytes interlaced with each other to form a confluent monolayer by stretching pseudopodia. (A) Magnification of 40 with phase contrast microscopy and (B) Magnification of 100. The figure represents three experiments.

rewarmed to 37 °C. Hereby, high cell yields and viabilities were obtained.

Figure 3. Beating rate of cardiomyocytes from 5 random fields was averaged on 3 DIV, 10 DIV and 20 DIV respectively. Data shows the means of quintuplicate determinations $\pm$ S.E. $p > 0.05$.

In view that deoxyribonucleic acid leaking from damaged cells during preparation will increase viscosity and lead to handling problems, we introduced purified deoxyribonuclease in the repeating digestion procedures to prevent accumulation of sticky DNA released from damaged cells. The yield of cells can also be increased by the presence of low amount of DNase I in trypsin solution. In addition, in the present protocol, the cell yield was also increased to some degree by reserving the suspension from the first digestion. The majority of the suspended cells obtained after the first digestion were isolated cells, other than red blood cells which are obtained by repeated rinsing of the removed hearts and the minced tissue with PBS. Even there were some remaining red blood cells however, as they are suspension cells, they could be easily removed when the culture medium was changed.

Figure 4. Immunoperoxidase cell staining of cardiomyocytes with anti-sarcomeric α actin antibody as the primary antibody (1:600). Most (>95%) of the cells in the culture were positive for sarcomeric α -actin. The figure represents three experiments.

In the present study, we have also tested whether the cultured cardiomyocytes can respond to Angiotensin II properly by increasing protein synthesis, one of the indicators for cardiac hypertrophy. As shown in Figure 6, ^3H -leucine incorporation into cardiomyocytes was dose dependently elevated by Angiotensin II. This result indicates that the present improved protocol produces healthy cardiomyocytes that can be used to duplicate the hypertrophy model. Therefore, the time-saving protocol we presented here has its

Figure 5. Negative immunoperoxidase cell staining of cardiac fibroblasts with anti-sarcomeric α actin antibody as the primary antibody (1:600). The magnification of the figure was 100. The figure represents three experiments.

Figure 6. Effect of different concentrations of Angiotensin II (10^{-8} M, 10^{-7} M, 10^{-6} M) on ^3H leucine incorporation into cultured neonatal rat myocyte for 48 h. Cardiomyocytes were cultured in 24-well plates for 2 days and then 12 h before the experiment the medium was changed for the serum-free medium which was supplemented with insulin, transferrin, vitamin C and vitamin B12. Finally the following 48 h the cells were incubated with ^3H leucine ($1 \mu\text{Ci ml}^{-1}$) in the absence (control group) or presence of Angiotensin II. * $p < 0.05$ vs. control. Data were from at least 3 independent experiments performed in duplicate.

practical value. Such a cell preparation method would be useful for studying signal transduction mechanisms underlying cardiac hypertrophy as well as for identifying potential therapeutic targets.

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