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An Optimized Method for the Isolation Primary Cardiomyocytes from Neonatal Mice

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Abstract

Cultured primary cardiomyocytes have universally been used in the study of cardiovascular researches. Here, we offer an optimized protocol for isolation and identification of neonatal mouse cardiomyocytes. The main advantage of this technique over other methods is the high yield, steady cell viability and reproducibility. We can obtain 1×106 cardiomyocytes from 2-3 neonatal mice based on this method. Over the 90% isolated cells are cardiomyocytes, and cardiac fibroblasts and cardiac endothelial cells were removed as much as possible. In addition, we use only commercially available solutions and classical digestion enzymes, which is less-costly.

Keywords

Cardiomyocytes; Cell Isolation Techniques; Primary Cell Culture.

1. Introduction

Cardiomyocytes, the cardiac muscle cells, are the primary unit of the contractile apparatus of the heart. Cardiomyocytes are striated muscle cells, with short cylindrical shape and branching. The lack of cardiomyocytes may results in many cardiac diseases, and the cadiovascular disease (CVD) also will leads to the decrease of the cardiomyocytes. Cardiovascular disease has always been and will still be one of the top death causes in the world. According to the report from World Health Organization (WHO), ischaemic heart disease (IHD), which is one kind of CVD, was the top one of leading causes of death in 2000 and 2019 [1]. When IHD happens, such as myocardial infraction (MI), the decrease of oxygen and nutrition supply, which is caused by the decrease blood supply after MI, will gradually kill the cardiomyocytes of the area where MI happens, or even cause heart failure [2].

Cardiomyocytes account for about 49.2% of the total number of cells in ventricle part of the heart [3]. The decrease of the number of cardiomyocytes after IHD will more or less lead to the decline in cardiac function.

After acute MI, how to recover or greatly improve the cardiac function clinically of the patients remains a challenge, since it is generally believed that the cardiomyocytes of adult mammals have little potency on regeneration [4]. While some studies found out that neonatal mammal' s heart within the postnatal 7 days, such as mouse, can completely regenerate after apical resection or myocardial infarction, which means that neonatal mouse heart still have some capacity on regeneration [5][9]. In other words, neonatal mouse can be a good model animal for studying the mechanism of heart regeneration after injury. To start the study on the mechanism of neonatal mouse heart regeneration, we can do the in vivo or in vitro research.

Here, we offer an optimized methods of isolation of primary cardiomyoctes from the neonatal mice. This method can help answering key questions in the field of cardiovascular research, and give insights into the unique molecular of cell biology of cardiac muscle cells, for instance, addressing how some genes, proteins or other moleculars influence cardiac signaling pathway, transcription and

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expression. This technique enables many downstream applications for further in vivo studies on the mechanism of how cardiomyocytes regenerate. We refer to some previous reports[8][11], and through many attempts, we found a more reproducible and cost-effective method.

2. Materials and Methods

2.1 Animals

Neonatal (within 3 days after birth) C57BL/6 mice were obtained from the Animal Research Center of Jinan University. The body weight and physiological conditions of animals were similar. Animal experiments were approved by the Ethics Committee of Jinan University and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD, USA).

2.2 Reagents

75% alcohol, 2,3-Butanedione monoxime, BDM (Sigma), tryrisin (Gibco), collagenase typeII(Roche), Hank's Balanced Salt Solution (Servicebio), Dulbecco's modified Eagle's medium (Corning), Medium 199 (Corning), cardiac troponin T monoclonal antibody (Invitrogen, mab-12960), Alexa Fluor®488-conjugated AffiniPure (Proteintech, SA00013-1), fetal bovine serum (Gibco), horse serum(Gibco), laminin (Corning).

Washing solution (0.1011 g BDM dissolved in 50 mL D-Hank's). Trypsin solution (0.5 mL of 5% trypsin dissolved in 9.5 mL of washing solution). Collagenase solution (0.04g BDM and 0.015g collagenase typeII dissolved in 20mL of M199 medium). Cell culture medium (2.5mL of FBS, 5mL of horse serum and 9.5mL of M199 dissolved in 32.5mL of DMEM cell culture medium).

Table 1. Washing solution recipe

Reagent	Dosage
BDM	0.101G
D-Hank's	50 ml

Table 2. Trypsin solution recipe

Reagent	Dosage
5% Trypsin	0.5 ml
Washing solution	9.5 ml

Table 3. Collagenase solution recipe

Reagent	Dosage
collagenase typeII	0.015 g
BDM	0.04 g
M199 medium	20 ml

Table 4. Cell culture medium recipe

Reagent	Dosage
DMEM medium	32.5 ml
M199 medium	9.5 ml
Horse Serum	5 ml
FBS	2.5 ml

ISSN: 2414-1895 DOI: 10.6919/ICJE.202204_8(4).0020

2.3 Isolation of Primary Cardiomyocytes from Neonatal Mouse

1)Prepare washing solution and trypsin solution base on the formula above, and keep all solutions on ice.

2)The neonatal mice (within three days of birth) are wiped with 75% alcohol for whole body disinfection, then neonatal mice were euthanized immediately.

3)A small incision is made with scissors under the left armpit of the mouse for the exposure of the heart.

4)The hearts are extracted from the bodies and transferred to the first dish that filled with washing solution on ice.

5)The remained blood in the isolated heart is squeezed out with a tweezer, then the hearts are transferred to the second dish that filled with washing solution on ice.

6)The hearts are then opened with scissors for removing all the remained blood and moved to the third dish with washing solution on ice.

7)The hearts are minced into pieces and digested with trypsin solution at 4℃ over night with gentle agitation.

8)When the heart tissue fragments are aggregated on the second day, all the supernatant is removed and 8mL of collagenase solution is added for the secondary digestion.

9)The heart tissues are digested with collagenase solution under 37℃ water bath and 60rpm shaking for 15 minutes until there is no obvious tissues.

10)After digestion, the cell suspension is mixed thoroughly with a pipette and is filtered with a 70 μm filter.

11) After that, the cell suspension is centrifuged at 660 rpm for 5 minutes.

12) All the supernatant is removed and 4mL of cell culture medium is added to resuspend the cells.

13) The cells are first planted into a 10-cm cell culture dish, after 2 hours, the supernatant which containing most of the cardiomyocytes is transferred to a cell well plate, which is pre-coated with laminin in advance (1.5 x 105 cells/cm2).

14) The isolated cardiomyocytes are cultured at 37℃, 5% CO2.

2.4 Immunofluorescence Staining

1)Abandon the culture medium and wash the cells with PBS three times.

2)Add 250μL cell fixative solution (PBS containing 4% paraformaldehyde) to each well and incubate for 20 min, discard fixative solution.

3)Wash the cells with PBS, three times.

4)Add the blocking and permeabilizing buffer 5% BSA (preparations of 0.5% Triton X-100 PBS) for 1 h, discard the liquid.

5)Add primary antibody (cTnT) diluted in blocking buffer at 4℃ overnight.

6)Wash the cells with PBS for 5 min. Repeat three times.

7)Incubate the cells in secondary antibodies (AlexaFluor 488) diluted in blocking buffer in a dark environment for 1 h.

8)Wash the cells with PBS for 5 min. Repeat three times.

9)Incubate the cells in DAPI solution in a dark environment for 12 min.

10)Mount the slides by anti-fluorescence quenching agent.

11)Observe the cells by laser scanning confocal microscope. All manuscripts must be in English, also the table and figure texts, otherwise we cannot publish your paper. Please keep a second copy of your manuscript in your office.

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3. Results

3.1 Growing Situation

Basing on the protocol above, we isolated hearts from 16 one-day-old (P1) neonatal mice and finally obtain 12 well cells in 12-well plate. (2 mouse pups for obtaining ~106 cardiomyocytes)

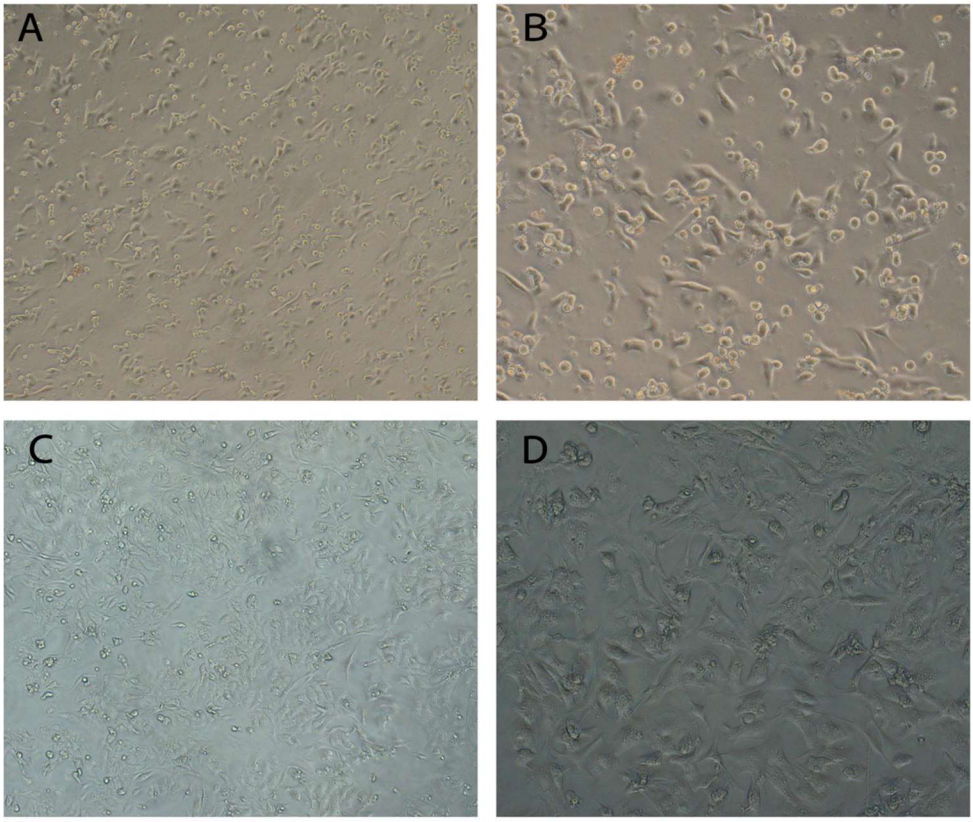

Figure 1. Culture of isolated primary cardiomyocytes of neonatal mice. A-B: After 24 hours in culture. C-D: After 48 hours in culture. After 24 hours, the primary cardiomyocytes were attached to the laminin coated culture wells and started to contract spontaneously. After 48 hours, the primary cardiomyocytes were stretched, and showed stronger contraction. The primary isolated cells were revealed as circular polygon and short cylindrical morphology.

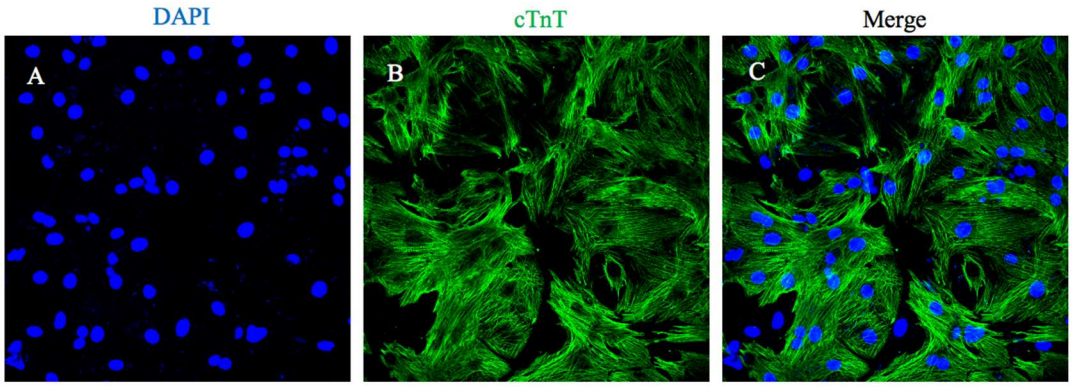

Figure 2. Immunofluorescence staining for primary neonatal cardiomyocytes. A: DAPI (Blue). B: cardiac troponin T (cTnT) (Invitrogen, Cat-No. mab-12960) (Green). C: Merged. Over 90% isolated cells were cTnT positive.

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4. Discussion

Cardiomyocytes have limited proliferative capacity in adult mammals. Newborn mice have the ability to regenerate about 15 percent of their ventricles within seven days of birth, and once this regeneration window has passed, the heart cells mature, and the mice permanently lose the ability to regrow damaged heart regions. [6] Thus, the neonatal mice we isolate are needed within postnatal 3 days, and it is better to use the P1 neonatal mice.

The main advantage of this technique over other methods is the reproducibility, high yield, and less-costly. As we mentioned above, 2 mice is needed for obtaining about 106 cells, which is higher than the previous methods [10]. We use only two classical enzymes for digestion, the trypsin and collagenaseIIwithout dispace. For retaining the balance between digestion and keeping cardiomyocytes alive, trypsin is used at 4℃ at the first night and collagenase typeIIis used at 37℃ for only 15 mins in the second day. The method mixing trypsin and collagenase do save a lot of time, but may easily over digest due to trypsin is strong at 37℃.

The BDM, a myocardial excitosystolic desensitization agent, protect the cardiomyocytes and prolong the survival time of cardiomyocytes. The reason is that BDM can inactivate calcium channels, reduce intracellular calcium concentration, and reduce calcium overload, thus preserving high-energy phosphate compounds.[7] Compared with previous protocols[8], we use less BDM, half of the previous protocols, but we can also maintain the cell viability.

The purification of cardiomyocytes from cardiac fibroblasts and endothelial cells is also the key of this experiment. Cardiomyocytes need longer time for adhering the dish, and also need the collage such as laminin coated. We obtained the supernatant which containing most of the cardiomyocytes after 2 hours, and at this time the fibroblasts and endothelial cells had adhered to the uncoated cell-culture-dish. The exact time of separation can be adjusted. There are some protocols show that 1-3 hours is the optional period[8]. When the cells is relatively exceeded the number we expected, the better measure is that absorb the supernatant after 1 hour, and then re-separate the cells for another 1 hour. It would be effectively decrease the rate of cardiac fibroblasts and endothelial cells. Based on this optimal methods, over 90% isolated cells are cardiomyocytes.

We replaced L-15 culture medium with M199 culture medium and found that the addition of horse serum to cardiomyocyte culture medium significantly increased cell viability.

In terms of specific markers of cardiomyocytes in immunofluorescence staining, cardiac troponin (Tn) is a regulatory protein of muscle tissue contraction, which is located on the thin filament of contractile protein and plays an important role in the regulation of muscle contraction and relaxation. The cTnT is specific to cardiomyocytes. And α-actin, MEF2C and PCM1 are also the unversally recognized cardiomyocyte-specific antigen.

Acknowledgments

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