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Research Article

Optimizing Cell Culture Strategy for H9c2 Cardiomyoblast: A Lesson Learned for Building Cardiovascular Disease Model

Muhamad Rizqy Fadhillah[1], Wawaimuli Arozal[2*], Heri Wibowo[3]

[1] Master Program, Biomedical Science, Faculty of Medicine, Universitas Indonesia
[2] Departement Pharmacology and Therapeutic, Faculty of Medicine, Universitas Indonesia
[3] Integrated Laboratory, Faculty of Medicine, Universitas Indonesia

[*] *Corresponding author: wawaimuli.arozal@ui.ac.id*

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Abstract: Cardiovascular disease become one of the leading factors of death in the world. Thus, research is urgently needed to discover newer drugs or therapeutical agents and biological plausibility. The H9c2 originated from embryonic BDIX rat ventricular cells and was previously used in numerous in vitro studies because of its similar nature to cardiomyocytes. However, in the present investigation, there are limited studies that specify the basic procedure for culturing H9c2 cardiomyoblast and arranging the best strategy for a suitable timeline. Here, we shared our experience in culturing the H9c2 cardiomyoblast, including harvesting and subculturing the cells. We also demonstrated the change of cell confluency, depending on the seeding number, serum concentration, and culture flask through days 1, 3, and 6, to determine their doubling-time population. H9c2 cardiomyoblasts doubling time is around 48-54 days with Mean±SD 2.38±0.41. However, seeding density, different culture flasks, and serum concentration have become independent factors in determining specific measures to harvest the cells for further experiments.

Keywords: Cell-line, Cell Culture, Cardiovascular Model, In-vitro

Introduction

Cardiovascular disease become a top ten contributor to higher mortality in the world. This could result from metabolic, vascular [1], or aging-related disease [2] reported in increasing cases in two decades [1] The integration of various fields is needed to increase the gap in advancing the research process on these subjects, such as candidates of an agent of therapy, diagnostic, or prognostication tools.

Preclinical research is a crucial step in each subject in cardiovascular medicine, especially finding newer agents or repurposing presence agents for modulating disease progression. To advance these demands, the need for predictive nonhuman models is urgent. Nowadays, some 3D cardiac tissue models that accurately mimic real human tissue are being manufactured. Another example that might be an option to mimic human tissue is pluripotent stem cells and primary neonatal cardiomyocyte rats (PNCM). However, high costs and relatively difficult optimization have become major problems for researchers using these cells. Even though PCNM is a reliable cell model for proposing many cardiovascular studies, the major disadvantage of using these cells is sacrificing more animals, which has become a worldwide concern.

H9c2 is a cell line (infinite cell line) harvested from BDIX embryonic ventricular cells of the rats. Despite the emergence of the updated cardiomyocyte model, H9c2 has been widely interested and used in cardiovascular studies, especially in cardiac hypertrophy [3–8] and toxicological analysis [9]. According to Watkins et al., these cells have proven similar responses to the PCNM for hypertrophy studies even though they originated from multipotent cells, raising questions about whether they might mimic the differentiated cardiac muscle cells. However, these drawbacks are compensated because the H9c2 can differentiate when the serum is reduced to 1% and supplemented with retinoic acid. In addition, the H9c2 cell line also has the same metabolism and genetic

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features with differentiated cardiomyocytes rather than other cell lines, e.g., HL-1 cell line and atrial cardiac rat tumor origin). In other words, this proves that H9c2 cardiomyoblast has some upper hand as a simple and reliable tool in in-vitro cardiovascular studies.

However, to our knowledge, fewer available studies have detailed the culture methods for H9c2 and proposed a culture strategy for designing the cardiovascular disease model. Thus, to fulfill this need, we demonstrated our experience optimizing the culturing procedure of H9c2 cells using basic tools in our study. We hope this study will influence and give insight into further research into utilizing H9c2 as a preferable simple and easy-to-handle cell in advancing preclinical cardiovascular studies.

Materials and Methods

The H9C2 culture procedure was performed in the Integrated Laboratory, Faculty of Medicine, Universitas Indonesia. It has been approved by the Ethics Committee, Faculty of Medicine, Universitas Indonesia, with ethics number KET-1828/UN2.F1/ETIK/PPM.00.02/2023. The cells were obtained and transported from the Center for Academic, Innovation, Technology and Health Research, Padjajaran University (PAMITRAN UP).

As recommended by ATCC, for culturing the H9c2 cell line (ATCC-CRL 1446), we used a complete medium with components as follows: Dulbecco's modified eagle medium (DMEM) high glucose, 25 g/L with L-glutamine 4 mM and sodium pyruvate (Corning Cat 10-013-CV), Fetal Bovine Serum (FBS), (Corning Cat 35-010-CV), and penicillin-streptomycin (Gibco). Trypsin 0.05% (Gibco) and phosphate buffer saline were used to wash the cells for harvesting. The cells were cultured using a T25 or T75 flask. The cryopreservative agent made from 95% of the complete media with 5% dimethyl sulfoxide (DMSO) was used for cryopreserving cells. Some other devices to perform sterile culture protocol were the biosafety cabinet (Biofuge Stratos Thermo Scientific) equipped with micropipette 1,000, 100, 20, and 10 µL, incubator (Thermo Scientific I160), microscope inverted (Nikon) powered by BetaView internal software for image processing and centrifuge (Biofuge Stratos Thermo Scientific).

Before starting, the handwashing procedure should follow the World Health Organization (WHO) protocol, using a sterile lab coat, head cap, and sterile hand gloves. The biosafety cabinet should be sterilized cautiously using alcohol 70% and UV light. Sterilization should also be performed for micropipettes before use. Sterile tissue should be placed in the inner basic safety cabinet (BSC) to absorb spillage from the reagent. Lastly, place wet and dry garbage.

We used DMEM high glucose, L-glutamine, with sodium pyruvate for base media, incorporated with FBS 10%, and penicillin-streptomycin 1%. Before starting the procedure, a sterile syringe of 10 mL, a syringe filter of 50 mL, and a labeled falcon tube of 50 mL should be placed. The mixture was made using beaker glass (100 c) through this procedure: (1) add 44.5 cc base media, five cc FBS, and 0.5 cc penicillin-streptomycin (1%), (2) resuspense the mixture by up-and-down technique, (3) remove the mixture aseptically using a syringe 10 mL and syringe filter to the 50 mL falcon tube, and (4) this would result in 50 mL of sterile complete media with FBS 10% and penicillin-streptomycin 1%. Complete Media were stored properly at 2-8 ^{o}C for around a month.

Harvesting, Subculture, and Seeding Protocol

When the cells are around 70-80% confluence, the flask should be subcultured to the other flask or harvested for cryopreservation or treatment. The medium in the flask was removed and then washed with phosphate buffer saline (PBS) twice to remove the serum remnant. Afterward, the trypsin 0.05% was added to 1 mL of the T25 flask. The cells were incubated for 2-10 min in a CO_2 5% 37^{o}C incubator. After the cells were detached (the cells were circular in morphology), a complete medium with the same volume with trypsin 0.05% was added. The mixture of this suspension was then transferred to the conical tube. The flask could be washed

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once with PBS to ensure the remaining cells were detached from the flask and removed to the same conical tube. At this point, the cells can be counted using the formula 1 [10].

$$Viable\ Cells = \frac{Number\ of\ alive\ cells\ in\ the\ entire\ five\ quadrant\ of\ haemocytometer}{5}\ x\ DF\ x\ 10{,}000\ x\ suspension\ volume$$

(1)

DF : Dilution factor of trypan blue with suspension cells

The cell counting was performed using haemocytometry under bright field microscopy with 4x objective magnification using the trypan blue exclusion method, which utilizes the mixture of 0.04% trypan blue and cell suspension with a volume ratio of 1:1 (DF are 2). The cell death might be calculated using the same formula 2 [10], while for the viability percentage we used formula 3.

$$Death\ Cell = \frac{Number\ of\ Death\ cells\ in\ the\ entire\ five\ quadrant\ of\ haemocytometer}{5}\ xDFx\ 10{,}000\ x\ suspension\ volume$$

(2)

$$Cell\ Viability\ Percentage = \frac{Alive\ cells}{Alive\ cells + death\ cells}\ x100\%$$

(3)

The remaining suspension should then be centrifuged with 125 g for five min~~utes~~. After that, the supernatant was discarded and replaced with a fresh, complete medium. This new cell suspension will be seeded into a new flask for subculturing or treatment. Every cell that undergoes harvest and subculture procedure will increase their passage by one.

The cells were observed daily using an inverted microscope (Nikon). The determination of the time of harvest or subculture is based on the eyeballing observation of confluency. Confluency was determined based on the observation of three independent observers. The cells were passaged when reaching confluency around 60-80%.

Optimizing Seeding Number, Serum Concentration, and Cell Culture Flask to Determine the Doubling Time.

We also optimized the doubling time population for H9c2 cells using different seeding numbers, sera concentrations, and culture flasks with the same incubation. We used a seeding number lower than recommended by the European Collection of Authenticated Cell Cultures (ECACC) [11] i.e., 1-3x10,000 cells/cm^2 of the flask. We used cells with passages 9-11 to test our protocol. First, we prepared the cells with the same T25 flask and one group with T75. We counted the seeding number, total alive cells, and death rate percentage at the end of incubation time. The cell's confluency on 1st,-3r^{d}, and 6th day are recorded. The cells will then be calculated for population doubling time [12] using the formula 4.

$$DT = T \ln 2(Xe - Xb)$$

(4)

DT = Doubling Time (days)

T = Incubation time (days, hours)

Xe = The cell number in the end of incubation time (cells/ml)

Xb = The cell number at the beginning of the incubation time (cells/ml)

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After the doubling time collected, we test the data using descriptive data (mean±SD) if distributed normally or (median±IQR) if not distributed normally. The normality was tested using Shapiro-Wilk analysis or a histogram using SPSS 20.0 (IBM, USA).

Result and Discussion

The summary H9c2 cells cultured profile and morphology (confluency profile) is depicted in **Table 1 and Figure 1.** The healthy cell will appear spindle-like with single nuclei and like to appear in groups of monolayer cells. The cells also might appear as polygonal, spherical, and angular. Spindle cells might appear in parallel. Most cells appear as mononucleated cells, but Hescheler et al. [13] using DAPI staining, reported that some cells appeared as binucleated (4.3%) and trinucleated (0.35%). This difference might not be observed through simple light microscopy.

Table 1. The Change of Cells Confluency Each Day and Its Comparison with Total Number Cells Harvested and Percentage of Cells Death

Flask ID*	Cell Culture Description#	Culture Flask	Confluence Changes			Total Number Cells Harvested (Cells/mL)~	Percentage of viability (%)	Doubling Time (Day)	Mean±SD (Days)
			1st Day	3rd Day	6th Day				
F1	P9 FBS 10%, Seeding 125,000 cells	T25	10-20%	50-60%	70-80%	±250,000	99%	2.321	2.38±0.41
F2	P9 FBS 10%, Seeding 250,000 cells	T75	10-20%	30-40%	70-80%	±330,000	98%	3.021	
F3	P10 FBS 10%, Seeding 121,000 cells	T25	10-20%	50-60%	70-80%	±320,000	98%	2.008	
F4	P11 FBS 20%, Seeding 131,000 cells	T25	20-30%	60-70%	80-90%	±200,000	90%	2.723	
F5	P11 FBS 10%, Seeding 131,000 cells	T25	10-20%	60-70%	70-80%	±350,000	99.5%	1.998	
F6	P11 FBS 10%, Seeding 262,000 cells	T25	20-30%	60-80%	95-100%	±500,000	99%	2.220	

*The Flask ID were identified as flask 1, flask 2, etc.

#The flask was determined as passage number, FBS concentration, and seeding number

~The cells were suspended in the 3 cc consisting of 1 cc of trypsin 0.05%, 1 cc complete medium, and 1 cc of PBS.

The range of cell confluency changes is not different among the six groups (1st, 3rd, and 6th 10-20; 50-70; and 70-90%). However, for the T75 flask, because the surface area is higher than that of the T25 flask, the confluence on the sixth day is relatively the same as the third day of culture in the T25 flask. Regarding the number of cells harvested, the counted cells are relatively the same (250,000-350,000 cells/mL). The passage number also does not have a significant influence, rather than the seeding number (Group F), which results in 500,000 cells/ml at the end of incubation periods. Compared to other groups, cell culture supplemented with FBS 20% has a lower yield of harvested cells (200,000 cells/mL) and a higher death rate (20%). We also found that the doubling time of H9c2 cardio myoblast was around 48 h (Mean±SD, 2.38±0.41 days) and reached 60-80% confluency on the sixth day of incubation. Regarding the doubling time, our findings were comparable to the previous study (DT 48 to 54 hours), with a good distribution of DT in each flask and no difference in each passage [14–16].

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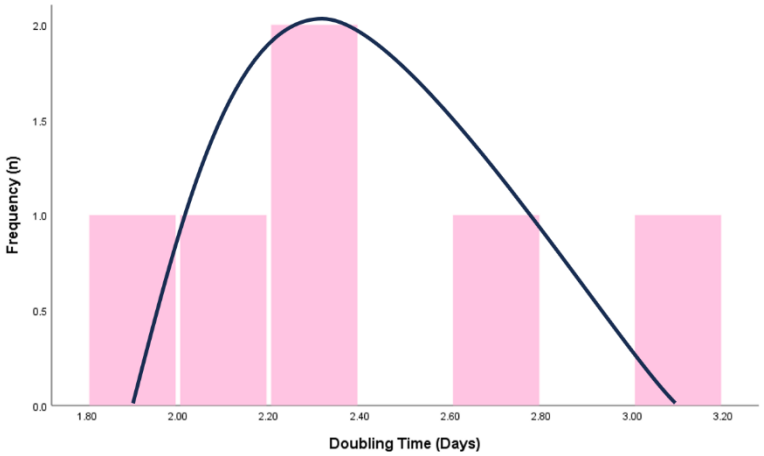

Figure 1 Histogram for Doubling Time Day Distribution for H9c2. p-value = 0.338

The H9c2 cardio myoblast culture procedure is relatively easy to perform. It has been validated in some previous research by Kankeu et al.[15] and Branco et al.[17], which confirmed the appropriateness of H9c2 use in cardiovascular studies through transcriptomic and proteomic analysis. Other things that might be considered are that the cost is relatively cheap, using a medium of standard ingredients according to the manufacturer's (DMEM, FBS 10%, and penicillin-streptomycin) without the addition of other growth factors, which is proven by the same doubling time as the previous study.

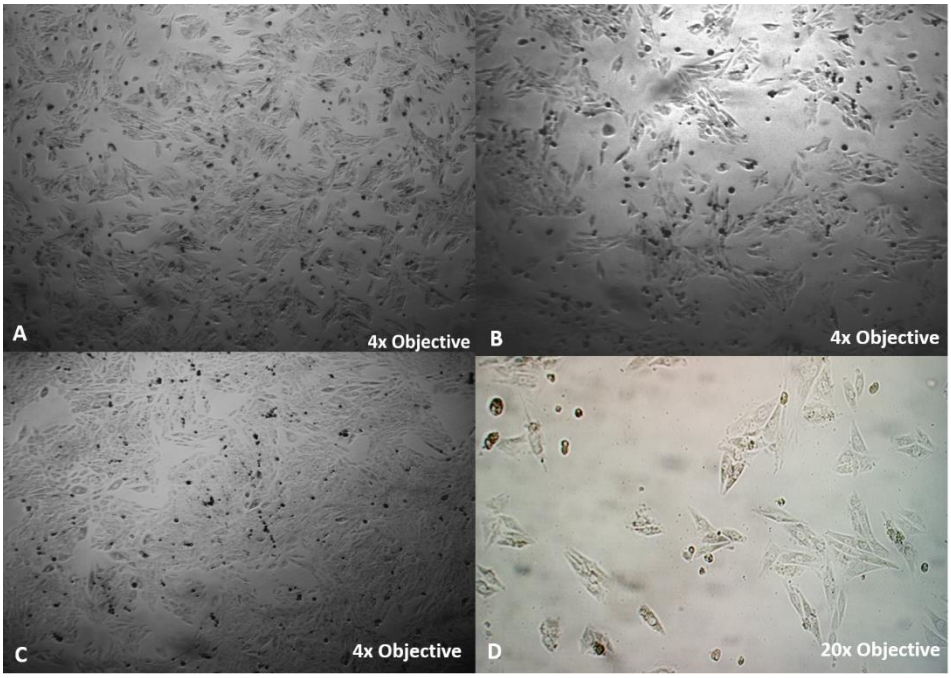

Figure 2. Representative Image of Confluency Analysis for H9c2 cells in 4x Obj. (A) Confluency of 80-90%, (B) Confluency 60-70%, (C), Confluency of 95-100%, (D) Morphological Analysis of cells in 20x Obj. The cells typically have a spindle-like appearance with one nuclei.

One feature that needs to be considered by other researchers is that H9c2 should be subcultured no more than 70-80% because this could disrupt the H9c2 differentiation capabilities [15,17]. Our experience found that

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the cells harvested in 70-80% confluency only reached 250,000 cells/ml for the T25 flask and 330,000 for the T75 flask. Some treatment procedures (e.g., protein analysis) need cells above this (6-well plate seeded around 600,000 to 1,000,000 for only one independent experiment) to yield good results. Since T75 has a three size of T25, T75 is preferred to perform massive experiments (three independent experiments) in one timeline, resulting in higher maintenance cost (three times medium volume needed).

Based on the ECACC datasheet [11], H9c2 is typically seeded in the 10,000-30,000/cm^2 flask for subculturing to maintain cell viability. In other words, the cells should be cultured in 250,000-750,000 in the T25 flask or 750,000-1,500,000 cells in the T75 flask. Our data suggested that when the cells reach 70-80% confluency, they will be harvested at around 250,000-330,000 cells/mL for six days. This means that the recommendation of ECACC will result in faster subculturing (2-4 days after confluency); this is also proven by flask 6, which showed 60-80% confluency on day 3. Using a two-week agenda, we compare the possibility of when the cells will be harvested using doubling time data from our study in **Figure 4** and **Figure 5 in the** T25 flask. Most of our T25 flasks are seeded by cells below 200,000, whereas ECACC uses above 200,000 seeding numbers.

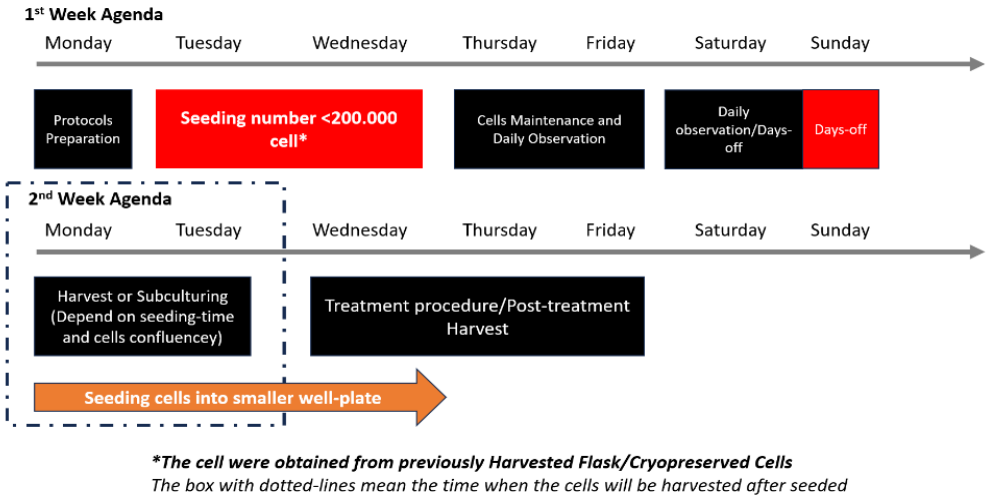

Figure 4 Proposed the culture strategy for determining the estimation time for performing treatment using seeding number <200,000 cells

The seeding cell below 200,000 showed a slower time to harvest than the seeding cell above 200,000 cells per flask (6 days vs 3 days, respectively). However, seeding cells below 200,000 cells becomes a safer option because the researcher probably will not harvest or subculture the cells on the weekend, and it is applicable for laboratories that are not operated on Saturday. Compared to seeding cells above 200,000, the researcher should harvest the cells on the weekend; even this condition might be compensated by performing protocol preparation and seeding the cells earlier in the first weekend. This also might become trouble if the laboratories are not operating on the weekend. Some studies also need to perform H9c2 differentiation (hypertrophy studies), which needs additional time to starve the cells by using FBS 1% [17]. Ultimately, if this protocol works, the treatment procedure might also be finished in the first weekend—much faster than the second protocol, and the second weekend can be optimized for biomolecular or proteomic analysis, compared to seeding below 200,000, which results in an additional week to perform. The first protocol might be suitable since our lab only operates on weekdays.

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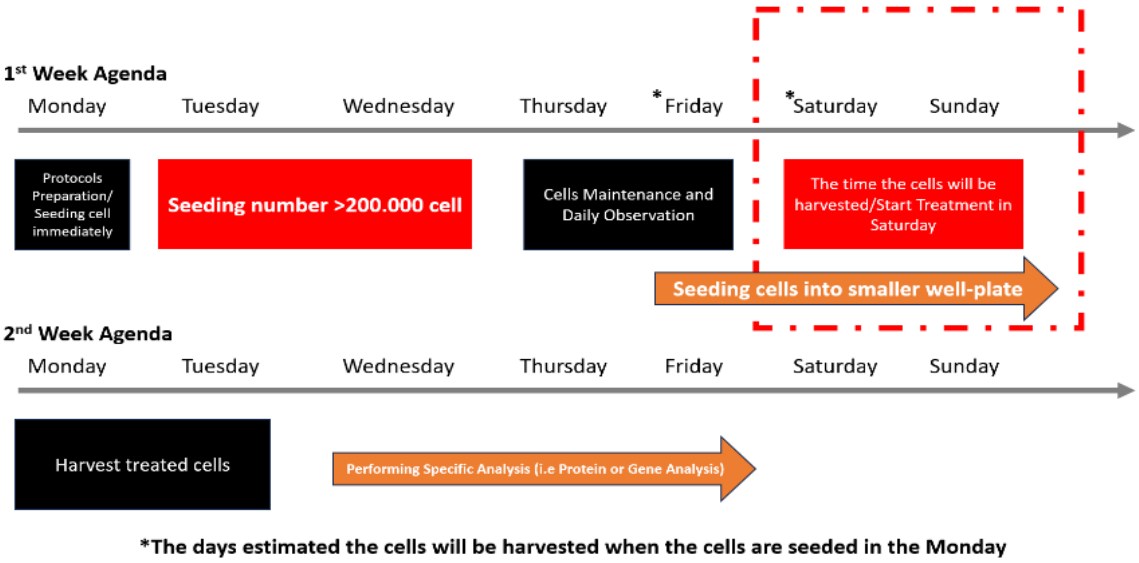

Figure 5 Proposed the Culture Strategy for determining the estimation time for performing treatment using seeding number >200,000 cells

Regarding the complete media, serum concentration needs to be adjusted and maintained in the range of 10% for routine culture for previous studies. ATCC recommended reducing the serum to 1% to induce cell differentiation, which was also performed in the published studies. Fetal bovine serum consists of adhesion molecules, antioxidants, buffers, carrier proteins, and growth factors, which are important in sustaining cell growth [18,19]. However, to our knowledge, we did not find any resource that stated how higher FBS concentration might affect the H9c2 cardio myoblast growth. Our preliminary findings suggest that the same seeding number with a higher concentration FBS (20%) might prolong the doubling time (1.998 vs 2.723 days) and have lower percentage cell viability (99.5% vs 90%).

Even though FBS supports the basal growth of many cells, it does not mean that increasing concentrations of FBS are linear to the increasing rate of growth or cell proliferation. Different compositions of FBS might vary according to the age, sex, physiological condition, and nutritional profile of animal blood where the serum is harvested.[20] Furthermore, FBS came with many brands, suggesting differences in their constituent because of different processing. Liu S et al. investigated eight different FBS brands affecting reproducibility in in vitro tests. They found that some FBS brands might stimulate the tested cells to produce specific cytokine and interleukin-8 and have varying regulation of metabolites [21]. Another issue is related to the extravesicular and other aggregated protein or growth factors reported to improve the cell's growth if removed [22]. From our perspective, the cause of the drop in doubling time and increasing rate of cell death are not merely caused by increasing concentration of FBS; they are supposed to be multiple issues that have not been further explored. On the other hand, FBS might need to be replaced somewhat with other compounds, for example, SH-SY5Y neuroblastoma cell, which might be replaced with Nu Serum (NuS) with a low concentration of protein to increase cell adherence [23].

The H9c2 cardiomyoblast cell culture procedure is likely the same as other cell lines and should typically be performed as the manufacturer instructed. Choosing the optimum type of basal media, FBS, and other growth factors or additive components to supply the cell's growth might be tailored to the need, and laboratory experiences in culturing other cell lines should be applied. Our results in this paper try to elaborate an easy and cheap protocol to maintain and decide the cell needs for further experimentation. This study warrants

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confirmation through biomolecular study to further see their reproducibility and acceptability in another laboratory.

Conclusion

It can be concluded that the H9c2 cardio myoblast cell line is a suitable model of cardiomyocyte for in-vitro studies, as it is easy to handle and relatively cheap. However, precautions for harvesting should be taken regarding choosing the FBS brand and keeping the blast cells' capabilities to differentiate for making good mimics to the differentiated human cardiomyocytes for higher quality cardiovascular in-vitro study models.

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Conflict of Interest

All author affirms no conflict of interest.

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