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# Hypertrophy Determination of H9c2 Cardiomyoblast Cell Line Using Wright-Giemsa Staining: An Experience in Developing an Acceptable and Easy-to-handle In-vitro Protocol

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## ABSTRACT

### Introduction
Cell-size area (CSA) becomes the standard parameter routinely tested in vitro for cardiac hypertrophy studies. Thus, staining is an essential tool for this purpose. As reported in a previous study, immunofluorescence staining is an established method for CSA. However, because it is expensive and requires a specialized microscope, e.g., immunofluorescence or confocal microscope, it is not applicable in a laboratory with limited equipment. Wright-Giemsa staining is a standard procedure in hematology laboratories and is inexpensive and convenient. Here, we shared our experience developing a CSA determination protocol using Wright-Giemsa in H9c2 cardiomyoblast.

### Methods
The viability tests were performed on H9c2 to determine the effective dosage of angiotensin II and Irbesartan (standard drug). The H9c2 were divided into three groups: the control group (without either angiotensin II or irbesartan), the negative control (with angiotensin II), and the positive control (with angiotensin II and Irbesartan), triplicate for each group. The cells then are acclimatized overnight, serum-starved for one day, and incubated with angiotensin and irbesartan for 48 hours. Lastly, Wright-Giemsa was observed using a light microscope in three fields. The CSA was determined by three independent observers blindly, statistically different if the $p<0.05$ using ANOVA ways or Kruskal-Wallis.

### Results
After the H9c2 induced by angiotensin-II 1 µM and Irbesartan 1µM, we found the CSA significantly differed among each group ($p<0,0001$). The negative control has a higher median and interquartile range (IQR) CSA (10.78 (6.79) $um^2$) compared to the control group (median (IQR) 7.27 (4.91) $um^2$) and positive control (median (IQR) 7.849(5.31) $um^2$).

### Conclusion
It can be concluded that the Wright-Giemsa might help determine the CSA for in-vitro hypertrophic studies.

**Keywords:** Cell-size area, H9c2 Cardiomyoblast, Wright-Giemsa staining

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## INTRODUCTION

Heart failure has still become a global topic and might further increase its mortality and morbidity. Around 1-2% of the world population suffering heart failure.[1] Furthermore, it is estimated that around 10% of the Asian population has heart failure, especially in Southeast Asia and Indonesia.[2,3]

The European Society of Cardiology (ESC) working group has recommended some heart failure drugs, sodium-glucose transporter 2 inhibitor (SGLT2 inhibitor), for treating diastolic heart failure, which previously did not have specific drug recommendations. However, mortality and morbidity did not reverse totally.[4,5] Hence, the drug discovery process must be accelerated.

Preclinical studies are one of the important steps in determining biological activity-specific products through specific targets or mechanisms to provide biological plausibility insight. Pathological cardiac remodeling is a pathophysiology underlying heart failure[6] and might be investigated through in vitro or in-vivo experiments.[7]

One of the important indicators in cardiac remodeling is cardiac hypertrophy of cardiomyocytes. Hypertrophy is the increase in the size of a cell surface or cell size area (CSA). This condition might be tested by calculating the area of each cell. Some techniques have been published, such as using flow-cytometry to sort the cells according to their area,[8,9] immunofluorescence to increase the visibility of the cells through confocal or immunofluorescence microscope, and mRNA Fetal gene expression, e.g., natriuretic peptide. Among those parameters, CSA is the most important parameter in cardiomyocyte hypertrophy.[10,11]

Hypertrophy can be tested either using primary cardiomyocytes or cell lines. One issue with using primary neonatal cardiomyocytes (PNCM) is the abundant use of rats, which increases the ethical issue. Furthermore, primary cardiomyocytes are relatively hard to handle and might use many resources. Another cell model that can be utilized is Human Inducible Pluripotent Stem Cells (HiPSC), but still, this one is also inexpensive and might not be applicable in every laboratory.[9]

H9c2 cardiomyoblast, a cell line from ventricular embryonic BDIX rats, has comparable results in hypertrophy response with PNCM. H9c2 is relatively easy to culture and differentiated by reducing the serum to 1% or adding retinoic acid. H9c2 is responsive to some hypertrophy inductors such as Angiotensin-II (Ang-II),[11–15] Endotelin-1 (ET-1),[10] High glucose,[16] catecholamine,[17] and many more.

CSA using Immunofluorescence methods is preferentially performed using wheat germ agglutinin (WGA)[18,19] or α-actinin[10] antibodies to increase the visibility of cytoplasmic compounds. One main advantage of immunofluorescence methods is that we might further apply the 3D aspect of the image. Thus, we would get a prediction of cell volume, which is much more sensitive than the calculated CSA.

Despite its superiority, immunofluorescence is not affordable to perform in every laboratory. It should be utilized using expensive tools, such as a confocal microscope, which is not affordable for some labs. Thus, alternative methods with nearly the same sensitivity, cheapness, and higher reproducibility are needed to face this condition.

Standard histological staining, e.g., Hematoxylin eosin and Wright-Giemsa,[20–24] is not new in determining CSA for hypertrophic studies. This kind of staining is relatively easy to perform with cheap materials. Furthermore, if the protocols are performed correctly, the results will increase the cell visibility for determining CSA. However, the previous study did not mention a complete or detailed protocol, for example, the tools used and how long the stain should be incubated, which can become practical issue in several labs.

To develop this acceptable and easy-to-handle method, we share our experience performing hypertrophic studies in H9c2 cardiomyoblast using Wright-Giemsa staining.

## METHODS

This research was done in the integrated laboratory, faculty of medicine, Universitas Indonesia and has been approved by the ethics committee of the medical faculty, Universitas Indonesia, with KET-1828/UN2.F1/ETIK/PPM.00.02/2023. The H9c2 cardiomyoblast (ATCC CRL-1446) was acquired from Pusat Akademik, Inovasi, Teknologi, dan Riset Kesehatan Universitas Padjajaran (PAMITRAN UP). We used Dulbecco's modified eagle medium (DMEM) with L-glutamine and sodium pyruvate (Corning Cat 10-013-CV), Fetal Bovine Serum (FBS)


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(Corning Cat 35-010-CV), and Penicilin-Streptomycin (PS) (Gibco) for cell culture maintenance. The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) (MTT), Ang-II, and Irbesartan were provided by SolarBio, MedChemExpress (HY-13984) and Sigma blue, eosin, and methanol are used from Wright-Giemsa.

First, the H9c2 cardiomyoblast is cultured using a complete medium of DMEM, FBS 10%, and PS 1% for seven days in a T25 culture flask. Whenever the cells reached 70-80%, we harvested them and subcultured them in the new flask.

Before start induction, the viability test was done to find the optimal dosage of Ang-II and Irbesartan to minimize their cytotoxicity effect and optimize the hypertrophic models. In this study, we choose Ang-II as a potent inductor proven by previous in-vitro study. Briefly, the cells were incubated with either Ang-II or Irbesartan for 48 hours, and further added MTT to determined their optical density. The optical density are transformed into viability percentage of each cells.

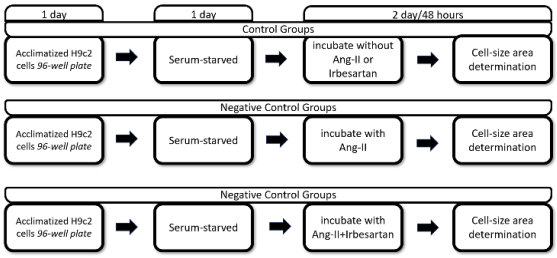


Figure 1. The H9c2 hypertrophic induction workflow.

The hypertrophy induction of H9c2 cardiomyoblast was briefly depicted in Figure 1**.** We divided the cells into three groups: (1) control group (cells without specific incubation substance), (2) negative control group (cells incubated with Ang-II only), and (3) positive control group (Ang-II with the addition of standard drug, Irbesartan), with each triplicate. After acclimatizing the cells overnight in a 96-well plate, we serum-starved them with complete media containing FBS 1% for one day. Each group and its triplicate incubate with Ang-II and/or Irbesartan for 48 hours.

After the inductions, the cells were then stained by Wright-Giemsa briefly explained in Figure 2. First, we removed the media. After that, the cells were washed with phosphate buffer saline (PBS) 2-3 times. The cells were then fixated with ice-cold absolute methanol (50 µL for each well) for 2 minutes and further air-dry the wells. We added 50 µL of methylene blue and incubated the well plate for 2 minutes. Further, the well plate is washed 2-3 times using distilled water. The 50 µL of eosin was added into each well, incubated for 2 minutes, removed, and then washed with distilled water 2-3 times. Lastly, the cells were observed in three fields in the light microscope using 20x objective magnification. ImageJ was used to aid the CSA calculation using the scale bar in the picture. In the ImageJ, "*Analyze*" > "*set scales*" and "*analyze*" > "*measure*" were used to determine the CSA through the selection of free hand tools. Three independent observers blindly determined the 450 CSA cells to test the results' validity.

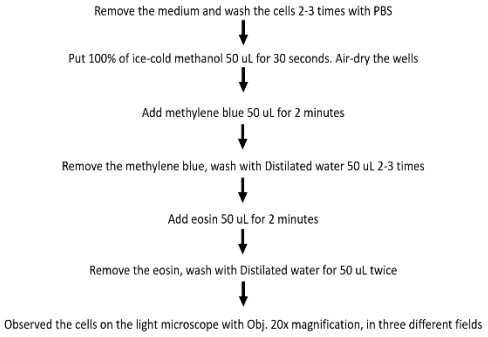


Figure 2. Wright-Giemsa Staining Protocol.

The CSA will be compared to each group, using either ANOVA Ways or Kruskall-Wallis. The difference will be statistically significant if the p-value is below 0.05.

## RESULTS

Our preliminary viability testing showed that Ang-II and Irbesartan are not affected the viability of H9c2 cells, thus we choose Ang-II 1 µM and Irbesartan 1 µM which frequently used in previous publication.

The Wright-Giemsa are represented in Figure 4. Our findings showed a significant CSA increase between the control and negative control groups ($p<0.0001$). Furthermore, after the cells were treated with irbesartan, the CSA of the positive control group was significantly reduced ($p<0.0001$) based on kruskall wallis analysis.


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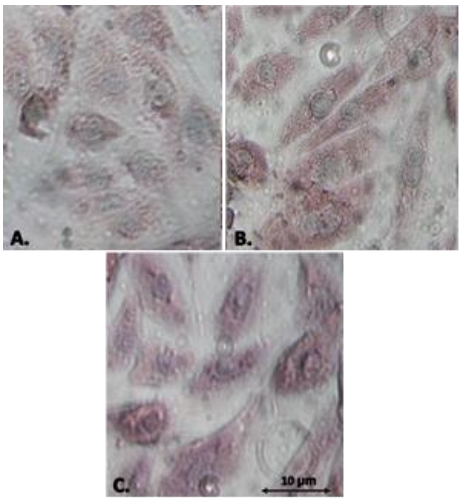


Figure 3. The Wright-Giemsa Staining Representative Image of Each Group. A Control Group. B. Negative Control. C. Positive Control.

To increase the validity of this study's results, we compare the data of 450 H9c2 CSA results among observers in the violin chart (Figure 5). The distribution of CSA among groups is not normal, but the median (minimum-maximum number) (Table 1) of CSA increases among the negative control group cell population. Also, the CSA in the positive group is remarkably reduced within a cell population.

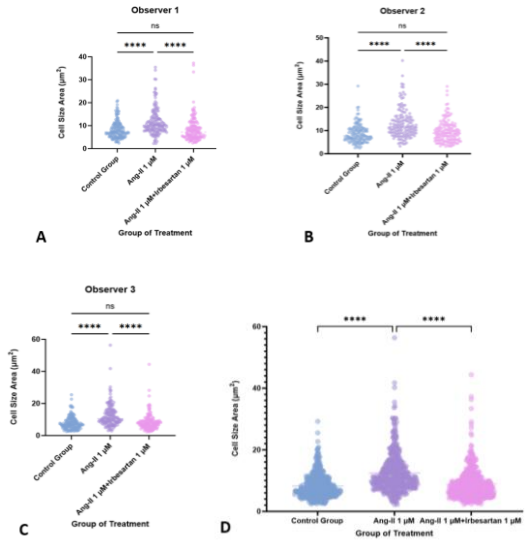


**Figure 4**. Violin Chart of CSA Distribution of (A) Observer 1, (B) Observer 2, (C) Observer 3, and (D) 450 Cells in total for each Group. ****p<0.0001

## DISCUSSION

The cell-size area or cross-sectional area of cardiomyocytes is one of the accessible indicators for determining cardiac hypertrophy, as it outlines the cells with visible sarcolemma and a central round of the nucleus. The CSA counting is performed manually or using specific software, such as CymoSize.[25] Good visibility and a better stain profile are needed to enhance the image profile.

Immunofluorescence probes, using specialized antibodies, such as WGA[19] from previous publications, are the most common tools that enhance the sarcolemma of the cells and create good surfaces or boundaries of cardiomyoblast cells, which might increase the precision of CSA counting. However, these techniques commonly need specialized tools that might not be reproducible in many labs.

Standard staining, such as Wright-Giemsa staining, is powerful yet cheap and easy. Wright-Giemsa (Romanowsky-type staining) is divided into two stages: methylene blue and eosin staining. Methylene blue (stains are known to stain the cytoplasm contents, whereas eosin stains the nucleus (blue to purple colors). Both combinations of these staining are powerful in identifying the morphology of the cells, especially cardio-myocytes. These standard staining are commonly performed in hematology labora-tories using proper techniques, especially for smearing and staining.[26,27]

In this study, we performed this staining directly on the well-plate after the Ang-II induced the cells with or without Irbesartan. The main principle of wright-staining is to incubate methylene blue and eosin after fixating the cell on absolute methanol. Because we first cultured the cells on the slide, we decided to fixate them in the well-plate and stain them in the same place. To our knowledge, no recent studies have reported how much fixative agent and staining (wright-Giemsa or another standard staining) we should put in each step of the well plate.[11,24] Routinely, the slides are just only dipped into the methanol and staining agent for a given time and washed with distilled water.[27]

To address this issue, we only add methanol, and the staining has minimal volume covering the entire well plate size. We use a 96-well plate, which has 0.32 cm2, and based on


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the information, the minimal volume that can cover up the entire size of the well plate is about 100 µL. We want to ensure that the stains go to all surfaces; we chose this volume for the fixating and staining steps. To rinse the methanol and stain, the distilled water is also 50 µL (below 100 µL).

From our protocols, the results were completely good. We did not find significant post-staining artifacts, yet the cytoplasm, nucleus, and background contour are differentiated. Our entire observation was good. Also, our findings showed that the CSA significantly increased the CSA among the three observers. Hence, we believe our protocols work and have good reproducibility.

Despite these good results, our protocol has significant limitation. Using methanol should be free of water because it might significantly create artifacts or bad-quality staining. Over drying should also be avoided, which is also one of the major causes of presence of bubbles.[27] As long as the researcher did not cautious with these things, the image quality might bad and limit the CSA calculation.

## CONCLUSION

It can be concluded that Wright-Giemsa staining might become another tool for increasing cardiomyocyte visibility to determine CSA.

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