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Article

Regenerative Potential of Injectable Platelet-Rich Fibrin to Accelerate Differentiation of Adipose-derived Mesenchymal Stem Cells into Cardiomyocytes-like Cells

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Abstract: Background: There is several challenges to solve irreversible loss of cardiomyocytes due to myocardial infarction. Cell therapy is believed as an ideal treatment for cardiac regeneration in the infarct area. Obtaining adipose-derived stem cells increases seems to be promising, however it is limited by the capacity to differentiate. Stimulation by injectable platelet-rich fibrin appears to have the beneficial effects to accelerate cardiomyocyte-like cells differentiation. **Objective:** To analyse the benefit of injectable platelet-rich fibrin to accelerate differentiation of adipose-derived mesenchymal stem cells into cardiomyocyte-like cells. **Methods:** This study is a true experimental randomized post-test design study. Adipose-derived mesenchymal stem cells were isolated from adipose tissues and cultured until 4 passages. The characteristics of adipose-derived mesenchymal stem cells were measured by the expression of CD 34-, CD 45-, and CD 105+ using flowcytometry. The samples were divided into 3 groups, i.e. negative control (α-MEM), positive control (differentiation medium) and treatment group (platelet-rich fibrin). The assessment of GATA-4 marker expression was conducted using flowcytometry on the fifth day and troponin was conducted using immunocytochemistry on the tenth day to determine the differentiation to cardiomyocyte. Data analysis was conducted using T-test and One-Way ANOVA on normally distributed data determined through Shapiro-Wilk test. **Results:** Flowcytometry on GATA-4 expression revealed significant difference on addition of platelet-rich fibrin compared with negative and positive controls (68.20 ± 6.82 vs 58.15 ± 1.23; $p<0.05$; 68.20 ± 6.82 vs 52.96 ± 2.02; $p<0.05$). This was supported by the results of immunocytochemistry on troponin expression which revealed significant difference between platelet-rich fibrin group compared with negative and positive controls (50.66 ± 7.2 vs 10.73 ± 2.39; $p<0.05$; 50.66 ± 7.2 vs 26.00 ± 0.4; $p<0.05$). **Conclusion:** Injectable platelet-rich fibrin has beneficial effect to accelerate differentiation of adipose-derived mesenchymal stem cells into cardiomyocyte-like cells.

Keywords: adipocyte-derived mesenchymal stem cells; cardiomyocyte-like cells; platelet rich fibrin; growth factor; stem cell therapy

1. Introduction

Coronary heart disease has a huge impact on age and quality of human life. Cardiomyocytes in adults have a limited capacity to regenerate after coronary heart disease.

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Where permanent damage to cardiomyocytes, loss of contraction function in the heart muscle and increased proliferation and turnover of fibroblast cells will lead to a progressive process of non-ischemic myocardial remodelling in the ventricles. And this remodelling process will cause progressive ventricular dilatation and lead to heart failure. Clinically there is no therapy that has the effect of regenerating myocardium in coronary heart disease. Therefore, cell therapy is the most ideal therapy for regeneration of damaged myocardium (1).

Bone marrow-derived MSCs (BMSCs) were the first cells to be recognized and most frequently studied in association with cardiovascular disease. Unfortunately, the use of BMSCs is invasive, painful, has a high morbidity rate and low success rate. On the other hand, adipocyte-derived mesenchymal stem cells (AMSCs) have an easier procedure and more promising results. It is said that AMSCs have better cell density than BMSCs (5% versus 0.1%) (2).

Growth factors play an important role in maintaining stem cell deficiency while blood derived growth factors and nutrients play an important role in stimulating stem cell proliferation. Platelets are the main source of growth factors that play a role in tissue regeneration. Platelet rich fibrin (PRF) is a new revolution in the concept of platelet therapy. Unlike platelet concentrates, this technique does not require a gelifying agent, but only centrifugation of natural blood without additives (3).

PRF contains a variety of growth factors. These growth factors trigger the movement, proliferation and differentiation of stem cells, such as in neovascularization and collagen synthesis. In addition, PRF also triggers soft tissue growth to accelerate healing and improve quality wound healing. Transforming Growth Factor-β1 (TGF-β1) triggers fibrosis while Platelet-Derived Growth Factor (PDGF) contributes to mesenchymal cell migration and survival. Insulin-Like Growth Factor-1 (IGF-1) prevents apoptosis, Vascular Endothelial Growth Factor (VEGF) stimulates vasculogenesis and angiogenesis and the function of Epidermal Growth Factor (EGF) in cell proliferation and differentiation (4).

Although several studies have been conducted that prove the role of platelets and the growth factors contained therein in the differentiation process, there is no study that discusses the effect of adding PRF to AMSCs differentiation, so in this study researchers wanted to find out whether the addition of PRF could have an effect on AMSCs proliferation through further observations.

AMSCs express markers of MSCs, namely CD10, CD13, CD29, CD34, CD44, CD54, CD71, CD90, CD105, CD106, and CD117. AMSCs do not express hematopoietic cell lineage markers such as CD45, CD14, CD16, CD56, CD61, CD62E, CD104, and CD106; and also endothelial cell lineages namely CD31, CD144, and von Willebrand factor. Morphologically, these cells are like fibroblasts and retain their shape after expansion in vitro. The similarity between AMSCs and BMSCs suggests that AMSCs originate from circulating BMSCs that infiltrate into the adipose compartment through the vessel wall (5).

2. Objective

This research aimed to analyse the benefit of injectable platelet-rich fibrin to accelerate differentiation of adipose-derived mesenchymal stem cells into cardiomyocyte-like cells, as expressed by level of GATA-4 and Troponin.

3. Methodology

Ethical approval

This research had been obtaining an ethical approval from Institutional Review Board of Dr. Soetomo Academic General Hospital - Faculty of Medicine, Airlangga University (Institution Review Board number: IORG0007195; Reference Number: 1733/KEPK/XII/2019) issued on December 27th, 2019.

Study design

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This research is an experimental laboratory study (in vitro study) with the administration of plasma rich fibrin (PRF) in AMSCs culture. Where this study aims to determine the effect of the addition of PRF on the ability of AMSCs to differentiate into cardiomyocyte-like cells. This type of research is true experimental with randomized randomization with a "post-test only control group design" approach.

Study setting

This research is a laboratory research that will be conducted at the Stem cell Laboratory, Centre for Biomaterials and Tissue Bank, Dr. Soetomo Academic General Hospital. This research was conducted for between 1 January 2020 - 31 December 2021.

Sample size

In this type of experimental research, there are 3 principles of processing research samples. Namely randomization, replication and control. In this study, these three principals were applied. Randomization was carried out before the research sample was divided into three groups. Furthermore, replication was carried out after the research sample was divided into three groups. The principle of replication is to increase the accuracy of research results with minimal sample variation. Where, when applied to the principle of statistical data processing, replication must be carried out at least three times, this is done so that the calculation of the standard deviation can be carried out. This is in accordance with the principle of Triplicate calculation. Then the addition of control is done with negative control and positive control.

Materials

1. Adipose-derived mesenchymal stem cells (AMSCs), namely mesenchymal stem cells (MSCs) obtained from minimal surgery procedures.
2. Bio Safety Cabinet class II
3. Centrifuge
4. Inverted Microscope
5. CO_2 Incubator
6. Pipette aid and micropipette
7. Water bath
8. Hot plate magnetic stirrer
9. Dissecting set
10. Vacuum pump
11. 500 mL Beaker glass
12. α-MEM medium
13. Phosphate buffer saline (PBS) with fetal bovine serum (FBS)
14. Triple express
15. Collagenase
16. $NaHCO_3$
17. Trypan Blue
18. 70% Alcohol
19. 15 mL and 50 mL conical tube
20. 5 cm dan 10 cm petri dish
21. 24 wells and 12 wells of microplate
22. Unit filter 0,22 μm (Millipore)
23. CD34, CD45, CD105 staining
24. GATA-4 antibody
25. cTnT antibody
26. Cardiomyocyte differentiated medium
27. Platelet Rich Fibrin

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Experimental procedures

This research was conducted through 5 stages, which can be explained as follows:

28. Isolation and culture of AMSCs from adipose tissue obtained from minimal surgery procedures
29. Identification of AMSCs from adipose tissue by Flow Cytometry by observing the expression of CD 105 and the non-expression of CD 34 and CD 45.
30. Culture of AMSCs on MEM alpha medium and differentiation of cardiomyocytes.
31. Preparation and administration of PRF in in vitro cultured AMSCs in treatment group
32. Evaluation of tissue culture of AMSCs in the three groups with flowcytometry for GATA-4 and Immunocytochemistry for cTnT

Statistical analysis

In this study, due to the number of samples < 30, the Shapiro Wilk test was used to test the normality of data. Data will be analysed inferentially using one way ANOVA if the data is normally distributed, or using the Kruskal-Wallis test if the data is not normally distributed. To analyse the differences between the two groups (post hoc) will use the T-test if the data is normally distributed, or Mann-Whitney if the data is not normally distributed. The limit of significance is $p = 0.05$.

Results

Isolation and culture of AMSCs

AMSCs were isolated from adipose tissue, extracted by minimally invasive surgical method. This isolation procedure is adjusted to the standard stem cell laboratory at the Stem cell Laboratory, Centre for Biomaterials and Tissue Bank, Dr. Soetomo Academic General Hospital. After isolation, AMSCs were cultured and expanded up to the 4th passage with the aim of maintaining the stemness properties of AMSCs and avoiding further cell differentiation. The majority of cells were seen with a spindle-like shape. In Figure 1 is a microscopic image of AMSCs culture starting from the time of isolation (passage 0) to the final culture (passage 4) (*Figure 1*).

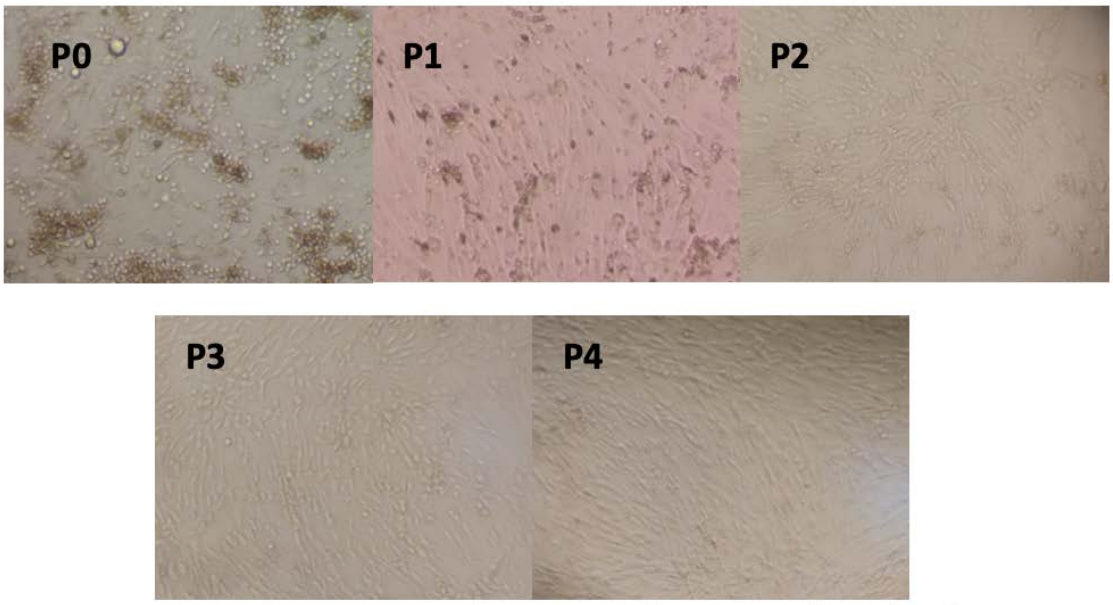

Figure 1. - AMSCs culture with 100x microscopic magnification from Passage 0 until Passage 4.

Phenotype Characterization of AMSCs

Based on the minimum criteria for the characterization of MSCs by The International Society for Cellular Therapy (ISCT), the majority of cells showed positive expression of

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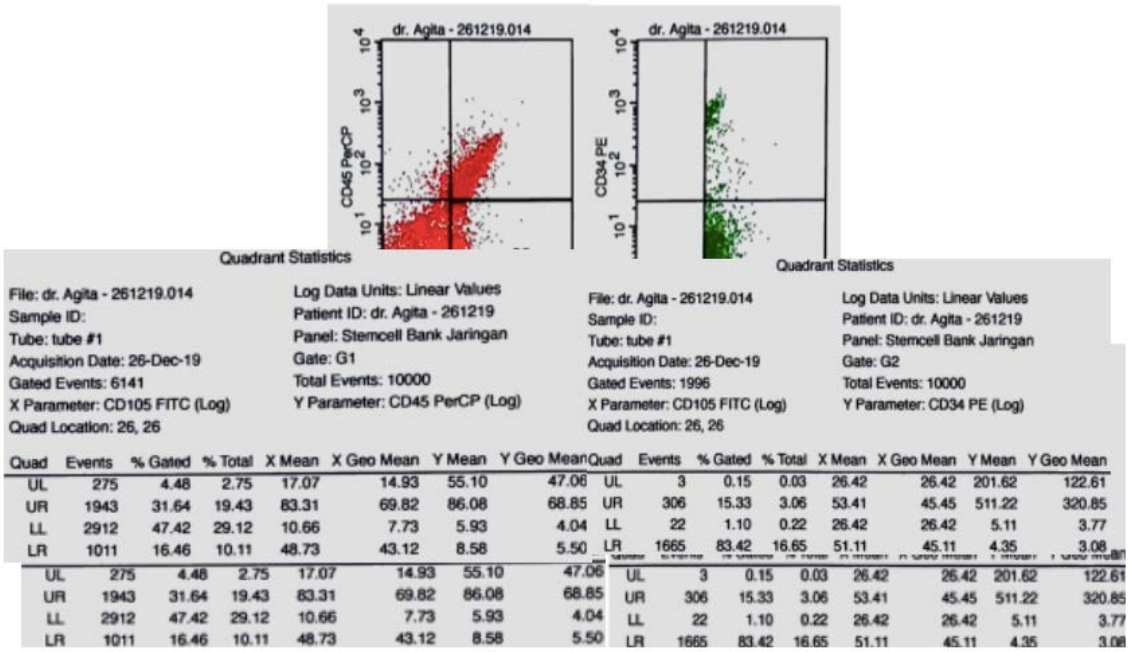
CD73, CD90, and CD105, and negative expression of $CD11_b$ or CD14, CD19, CD34, CD45, and HLA-DR. In this study, AMSCs that had been taken from adipose tissue were examined for characterization of MSCs in the 4th passage culture. AMSCs culture showed positive expression of CD105+ and negative expression of CD34- and CD45- by Flow Cytometry examination. Phenotype identification is based on cell morphology viewed by an inverted microscope (x100 magnification) (*Figure 2*).



Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	275	4.48	2.75	17.07	14.93	55.10	47.06
UR	1943	31.64	19.43	83.31	69.82	86.08	68.85
LL	2912	47.42	29.12	10.66	7.73	5.93	4.04
LR	1011	16.46	10.11	48.73	43.12	8.58	5.50

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	3	0.15	0.03	26.42	26.42	201.62	122.61
UR	306	15.33	3.06	53.41	45.45	511.22	320.85
LL	22	1.10	0.22	26.42	26.42	5.11	3.77
LR	1665	83.42	16.65	51.11	45.11	4.35	3.08

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	275	4.48	2.75	17.07	14.93	55.10	47.06
UR	1943	31.64	19.43	83.31	69.82	86.08	68.85
LL	2912	47.42	29.12	10.66	7.73	5.93	4.04
LR	1011	16.46	10.11	48.73	43.12	8.58	5.50

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LR	1665	83.42	16.65	51.11	45.11	4.35	3.08

Figure 2. - Identification of flow cytometry characteristics of AMSCs.

Assessment of GATA-4 differentiation marker cardiomyocyte-like cells differentiation with flowcytometry

GATA-4 examination at the cardiomyocyte-like cells differentiation stage was carried out on day 5 because AMSCs already appeared confluent. To find out the difference test between the three groups, it begins with a distribution test to determine the normality of the distribution of the value data. Data normality test was performed using Shapiro Wilk. The distribution of the data is said to be normal if the p value > 0.05, in this study the data was normally distributed with the p value = 0.118.

From the results of the flowcytometry examination of the three groups, the ratio data of GATA-4 (upper right quadrant) compared to the unexpressed GATA-4 (lower right quadrant) in the AMSCs group was an average of 58.1467 $\pm$ 1.23 in the negative control group (α-MEM) (Figure 3); 52.9633 $\pm$ 2.02 in the positive control group (medium of differentiation) (*Figure 4*); and 68.20 $\pm$ 6.82 in the treatment group (medium of differentiation + PRF) (*Figure 5*). This shows that the treatment group is superior to the negative control group and the positive control group is judged by the average ratio.

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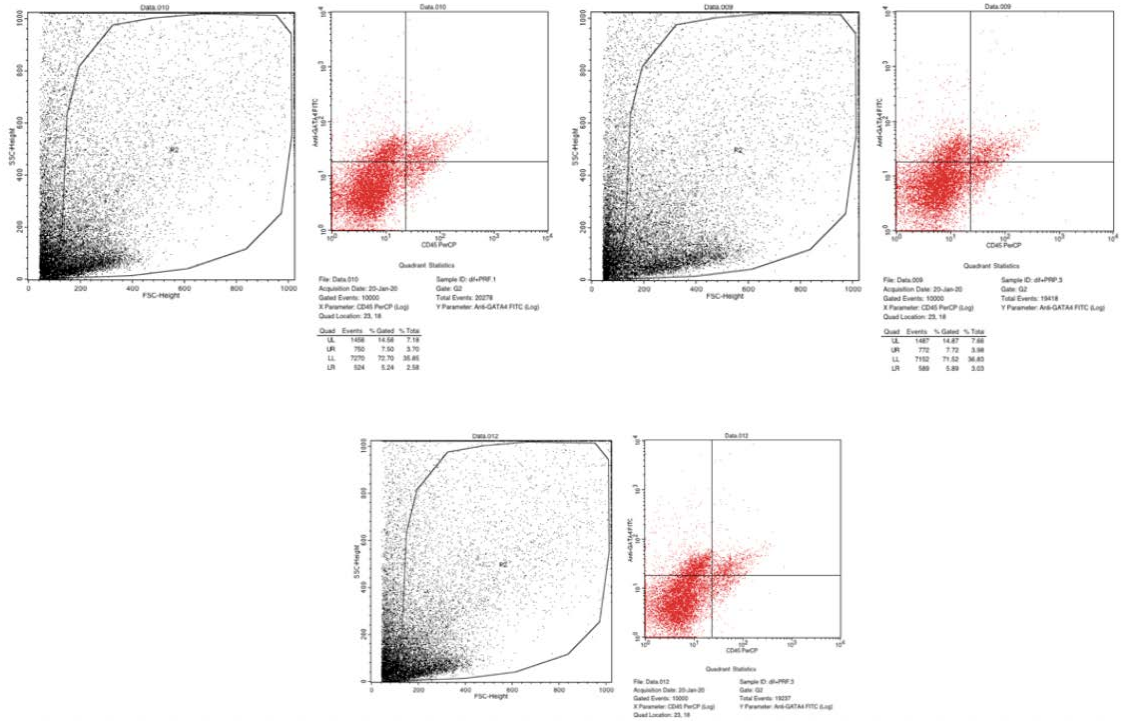

Figure 3. - Flowcytometry of GATA-4 in the negative control group (α-MEM).

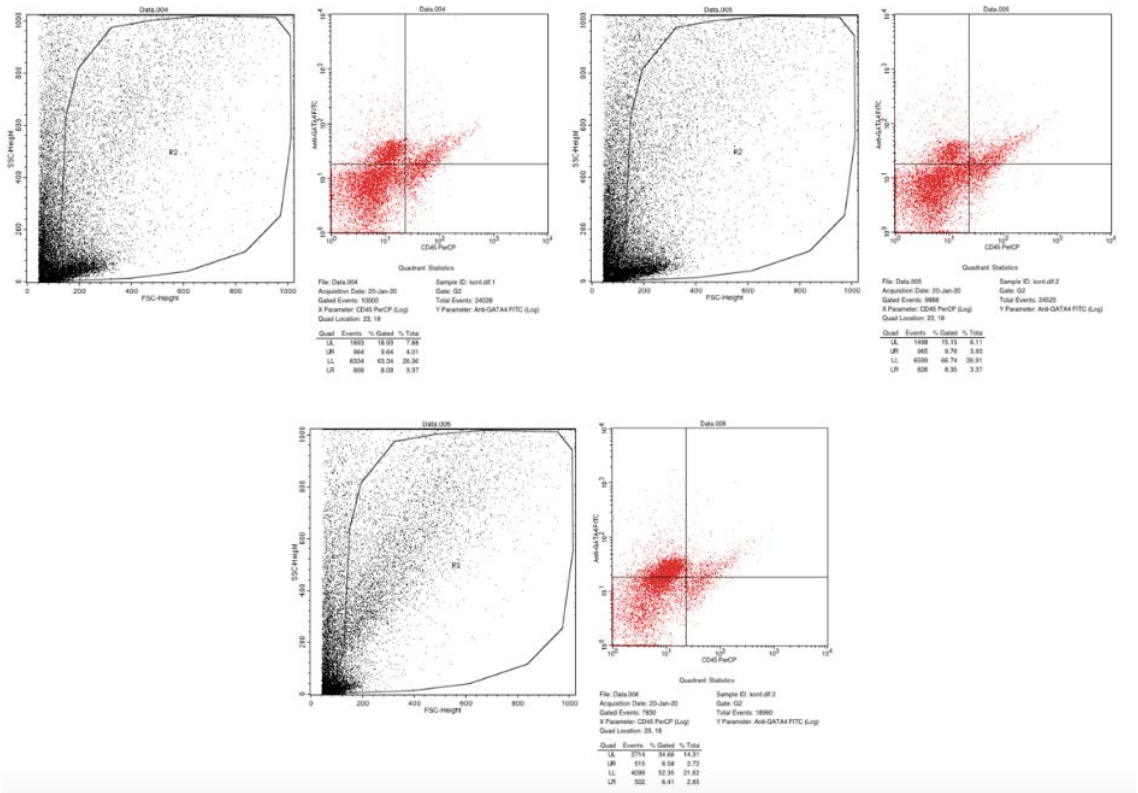

Figure 4. - Flowcytometry of GATA-4 in the positive control group (medium of differentiation).

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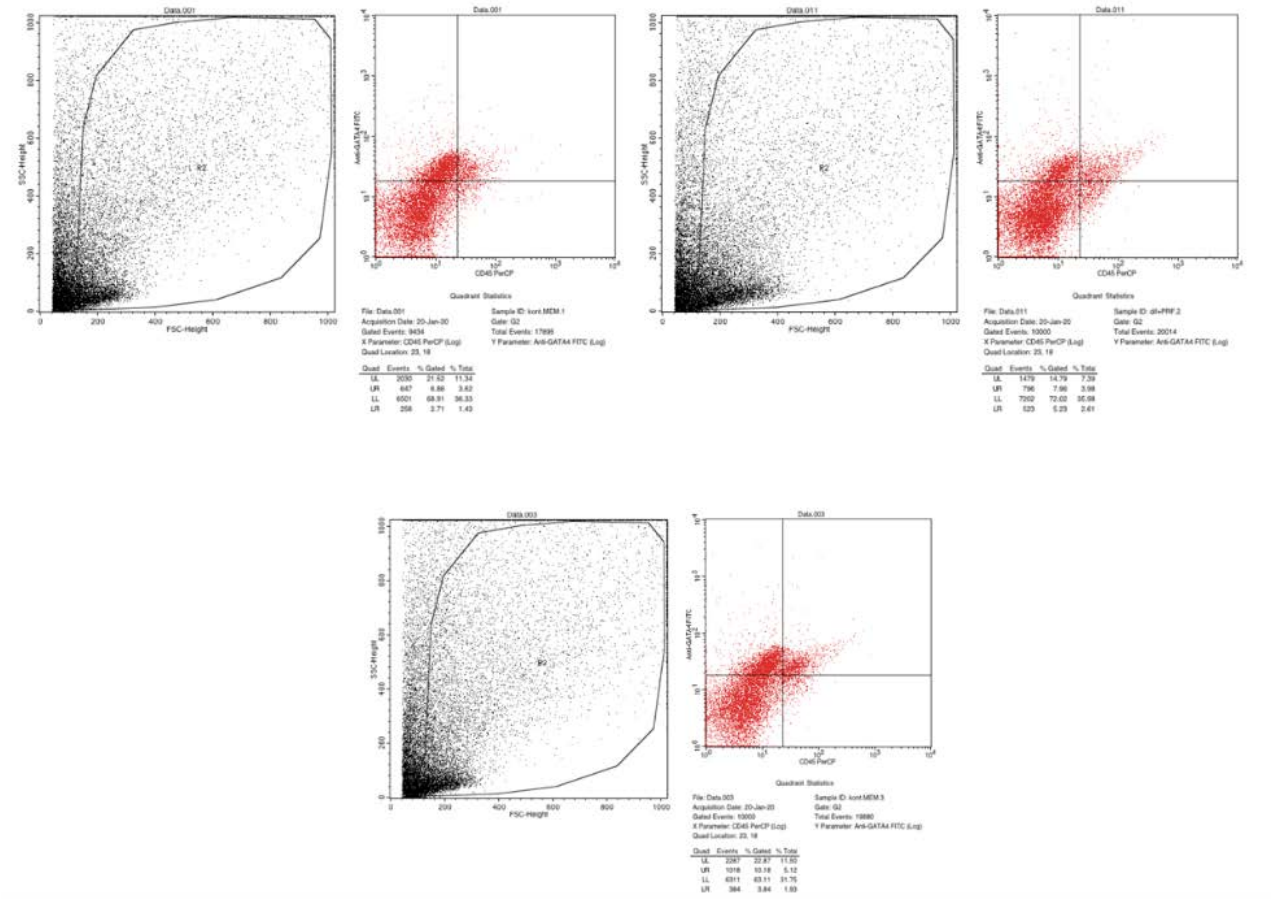

Figure 5. - Flowcytometry of GATA-4 in the treatment group (medium of differentiation + PRF).

Because the distribution of the data is normal, ANOVA test was carried out to determine the difference between the three sample groups with $p = 0.011$. This shows that there is a significant difference in the ratio of GATA-4 (upper right quadrant) compared to unexpressed GATA-4 (lower right quadrant) between the three groups. While the T-test was conducted to determine the difference between the two groups. There were significant differences between the negative control group (α-MEM) compared to the treatment group (differentiation medium + PRF) and the positive control group (differentiation medium) compared to the treatment group (differentiation medium + PRF) with $p < 0.05$. However, there was no significant difference between the negative control group (α-MEM) compared to the positive control group (medium of differentiation) with $p = 0.179$ (*Figure 6)*.

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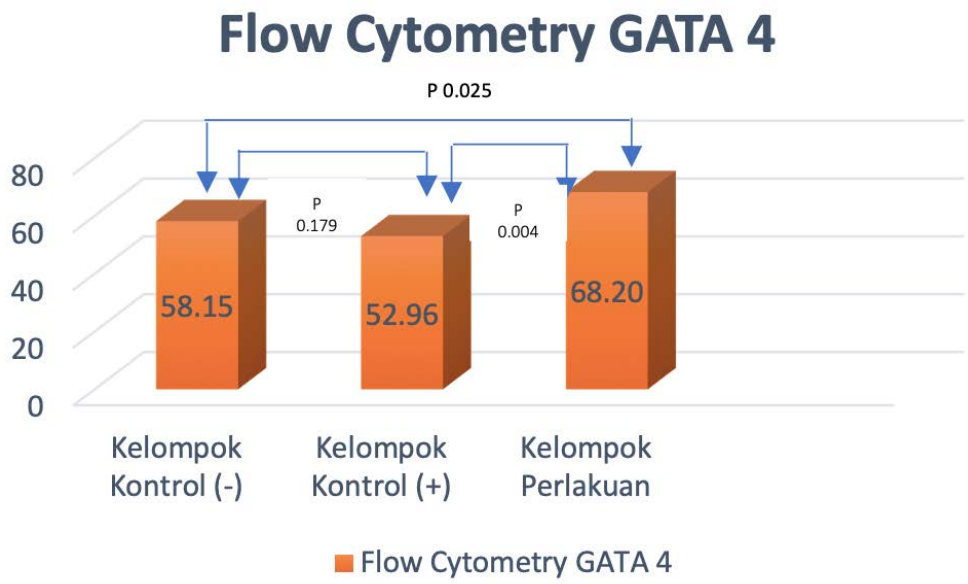

Figure 6. - Bar diagram showed the ratio of GATA-4 expression divided to unexpressed GATA-4 in the three group of AMSCs.

Assessment of expression of cardiomyocyte-like cells differentiation marker Troponin by Immunocytochemistry

Troponin (cTnT) is a late marker of cardiomyocyte-like cells that begins to be detected on day 10. On the day-10 of treatment, the three groups were assessed for immunocytochemical marker expression of cTnT. On immunocytochemistry examination, the determination of cardiomyocyte cells was morphologically visualized by staining the cytoplasm by DAB Chromogen and staining the nucleus by Meyer Hematoxylin. Description of cardiomyocyte-like cells appear more prominent than other cells, with prominent blue nuclei and prominent brown cytoplasm. In the three groups, the accumulation of cardiomyocyte-like cells per visual field was calculated. To find out the difference test between the three groups, it begins with a distribution test to determine the normality of the distribution of the value data. Due to the number of samples < 30, the data normality test used was Shapiro Wilk. The distribution of the data is said to be normal if the *p* value > 0.05, in this study the distribution of the data is normal with the *p* value = 0.315.

From the results of the immunocytochemistry examination of the three groups, the data obtained an average of 10.73 $\pm$ in the negative control group (α-MEM) (*Figure 7)*; 26.00 $\pm$ 0.4 in the positive control group (medium of differentiation) (*Figure 8*); and 50.6 $\pm$ 7.2 in the treatment group (differentiation medium + PRF) (*Figure 9*). This shows that the treatment group is superior when compared to the negative control group and the positive control group based from the mean cTnT expression.

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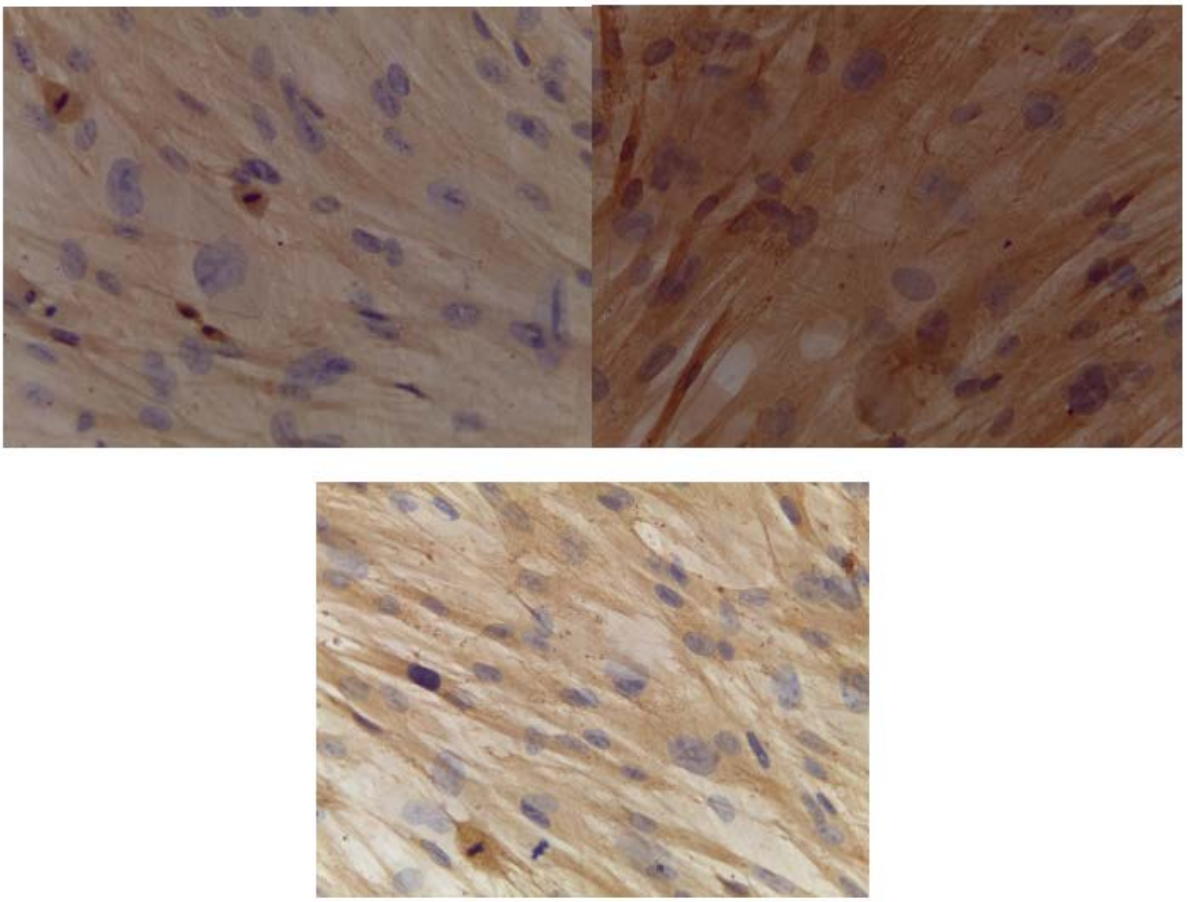

Figure 7. - Immunocytochemistry of cTnT in the negative control group (α-MEM) with 400x magnification.

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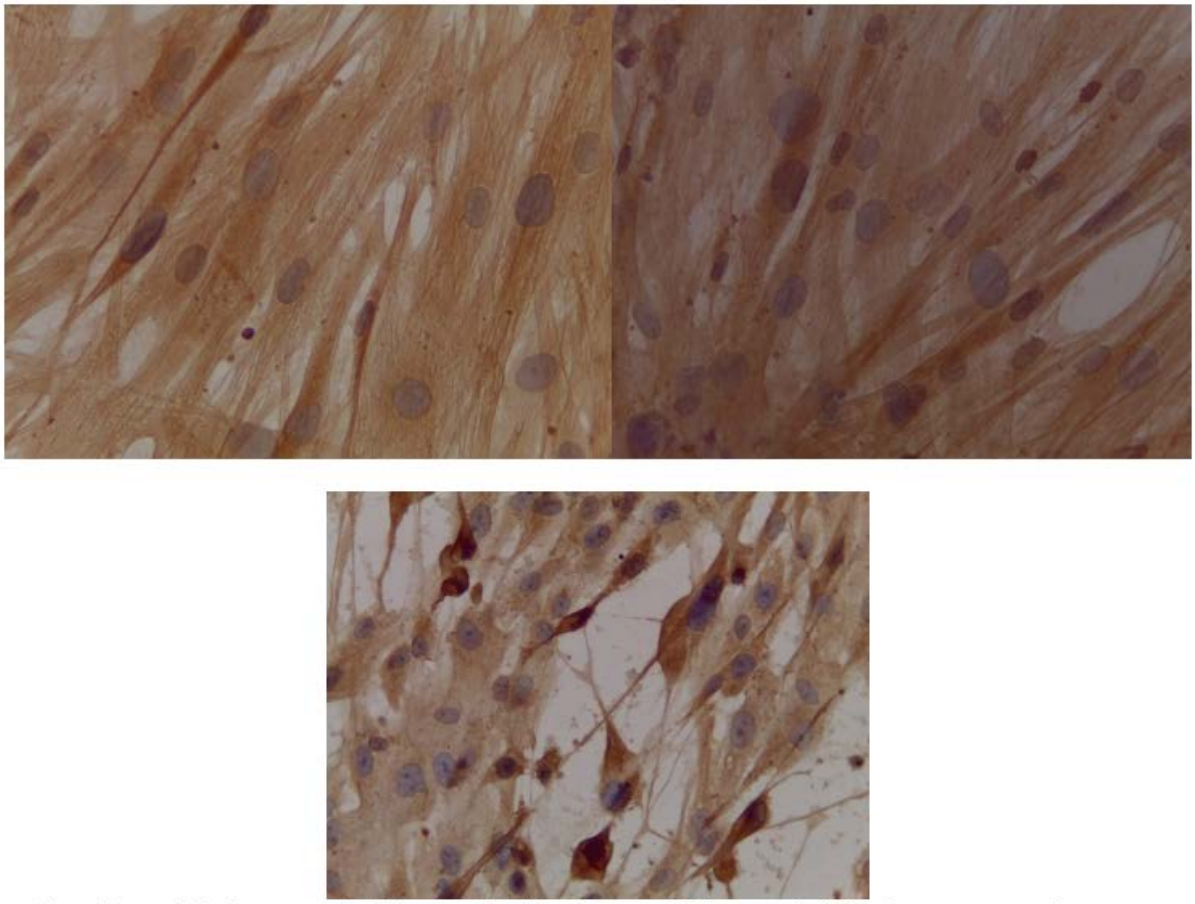

Figure 8. - Immunocytochemistry of cTnT the positive control group (medium of differentiation) with 400x magnification.

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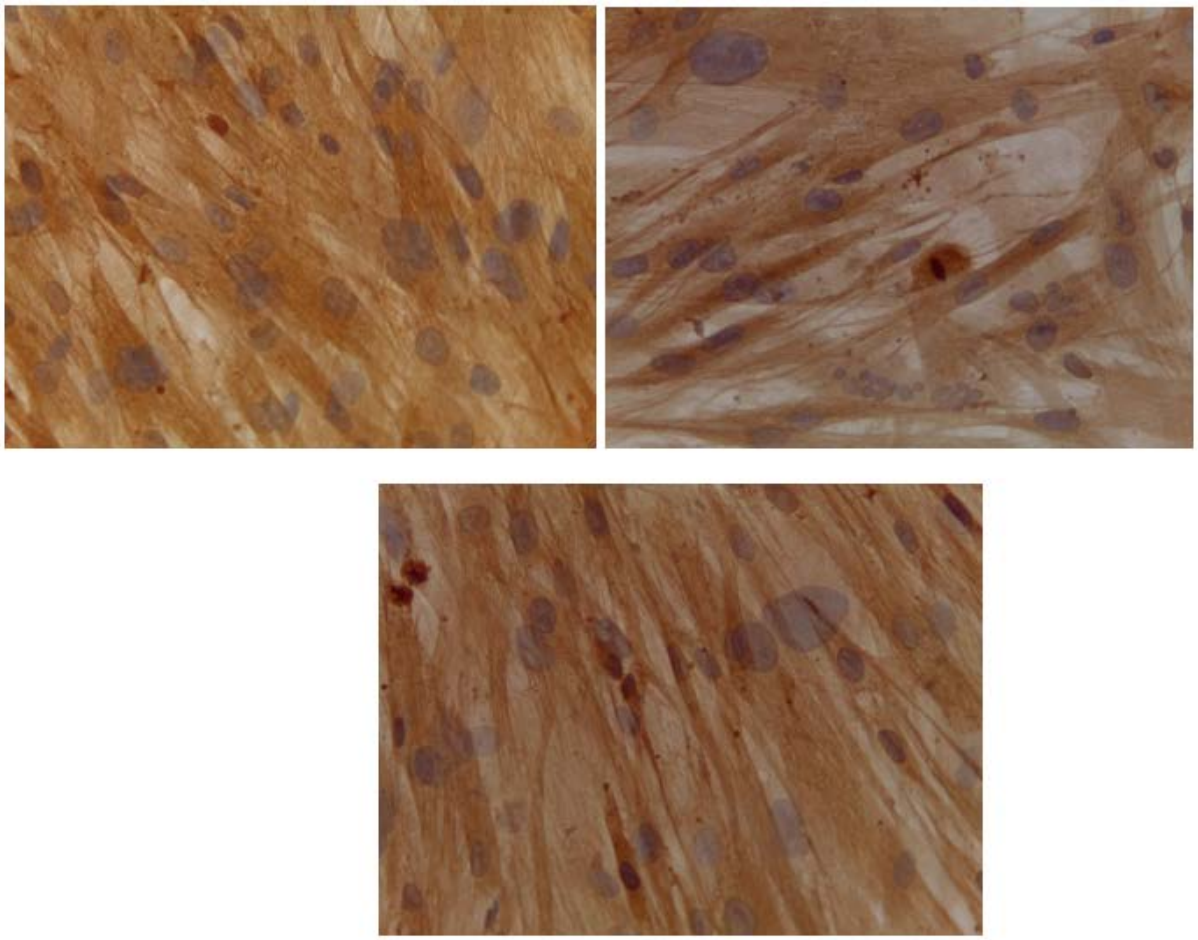

Figure 9. - Immunocytochemistry of cTnT in the treatment group (medium of differentiation + PRF) with 400x magnification.

Because the data were normally distributed, ANOVA test was carried out to determine the difference between the three sample groups with $p = 0.0001$. This shows that there is a significant difference in the mean of cardiomyocyte-like cells expressed with troponin between the three groups. While the T-test was conducted to determine the difference between the two groups. There were significant differences between the negative control group (α-MEM) compared to the treatment group (differentiation medium + PRF), the negative control group (α-MEM) compared to the positive control group (differentiation medium), and the positive control group (differentiation medium). compared with the treatment group (medium of differentiation + PRF) with $p < 0.05$ (*Figure 10*).

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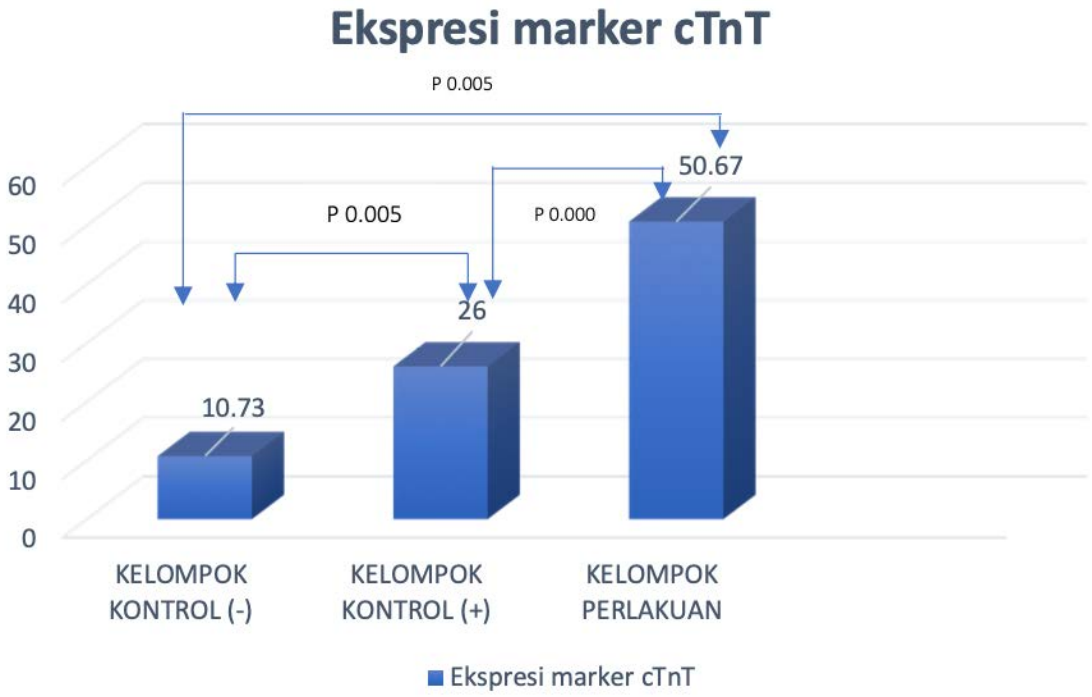

Figure 10. - Bar diagram showed mean cTnT expression by immunocytochemistry in the three group of AMSCs.

Discussion

In this study, two examinations were carried out at the stage of differentiation of cardiomyocyte-like cells. In the first examination, namely at the cardiac progenitors stage in the treatment group that assessed GATA-4 expression by flow cytometry, it showed a significant increase in the PRF group compared to the negative control group and the positive control group (68.20 ± 6.82 vs 58.15 ± 1.23 $p<0.05$; 68.20 ± 6.82 vs. 52.96 ± 2.02 $p<0.05$). And in the second examination, namely at the stage of mature cardiomyocyte-like cells assessing troponin expression with immunocytochemistry showed a significant increase in the PRF group compared to the negative control group and positive control group (50.66 ± 7.2 vs 10.73 ± 2.39 $p<0.05$; 50.66 ± 7.2 vs 26.00 ± 0.4 $p< 0.05$). This is in accordance with the research hypothesis which states that there is an effect of adding PRF to the differentiation of AMSCs into cardiomyocyte-like cells.

Adipose tissue is a potential source of adult stem cells that can perform multipotent differentiation. Where adipose-derived mesenchymal stem cells (AMSCs) have very similar properties to bone marrow-derived MSCs including the ability to differentiate into osteoblasts, adipocytes and chondrocytes under certain in vitro conditions. Similar to other mesenchymal stem cells, various studies have shown that AMSCs have the potential to undergo adipogenesis, chondrogenesis, osteogenesis, myogenesis, and vasculogenesis. Because MSCs have many surface antigens, the International Society of Cellular Therapy (ISCT) in 2006 determined the minimum criteria for defining the culture of MSCs, namely (a) having plastic adherent properties on standard culture media, (b) positive expression of CD73, CD90, and CD105 , and negative expression of CD11b or CD14, CD19 or CD79a, CD45, and HLA-DR, and (c) have the potential for differentiation into adipocytes, chondrocytes, and osteoblasts using staining in in vitro cell cultures. This study used the expression phenotype characteristics of CD105+, CD34- and CD45- on AMSCs cultured in Passage-4 by flow cytometry technique. This is in accordance with the minimum criteria for the characterization of MSCs presented by ISCT (6).

Coronary heart disease among other cardiovascular diseases has a very large influence on age and quality of human life. Cardiomyocytes in an adult have a limited capacity to perform regeneration after coronary heart disease. Where permanent damage to cardiomyocytes, loss of contraction function in the heart muscle and increased proliferation

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and turnover of fibroblast cells will lead to a progressive process of non-ischemic myocardial remodelling in the ventricles (7). And this remodelling process will cause progressive ventricular dilatation and result in heart failure. Clinically there is no therapy that has the effect of regenerating myocardium in coronary heart disease. Therefore, cell therapy is the most ideal therapy for regeneration of damaged myocardium (8).

Platelet rich blood derivatives have been widely used in various fields of medicine and stem cells for tissue engineering because of their consistent ability to increase the potential for proliferation, migration and differentiation of stem cells. Platelet rich fibrin (PRF) is a new revolution in the concept of platelet therapy. Unlike platelet concentrates, this technique does not require a jellifying agent, but only centrifugation of natural blood without additives (9-12). Platelets are composed of granules including alpha granules, dense granules, and glycogen granules. Alpha granules are the main granules that contribute to wound healing through the various growth factors contained in it. Growth factors present in PRF include platelet derived growth factor (PDGF), transforming growth factor- β1 (TGF- β1), vascular endothelial growth factor (VEGF), insulin like growth factor-1 (IGF-1), epidermal growth factor (EGF), fibroblasts growth factor and others (11).

The factor reaches the target cell and binds to transmembrane receptors and activates various intracytoplasmic proteins causing actions related to gene expression that have effects such as cell mitosis or collagen production. With the high content of growth factors possessed by PRF and the simplicity of manufacture, PRF can be an alternative for use in the field of cell therapy. Unfortunately, there are no studies linking the benefits of PRF with cardiomyocyte-like cells differentiation. This is what underlies this research. In previous studies, the optimum concentration of PRF varied from 50%, 10%, to less than 1%. Soffer et al, suggested a PRF of 0.5-1% as the optimum concentration for the rate of cell proliferation and mineralization (13). However, Ferreira et al, found that 2% PRF was the optimum concentration for osteoblast proliferation (14). In addition, Castegnaro et al. (2011) reported that 10% PRF was sufficient to induce cell proliferation in MSCs derived from adipose tissue (15). In the study of Govindasamy et al. (2011), 2% platelet lysate was referred to as the optimal concentration for mesenchymal stem cell and dental pulp stem cell proliferation and osteogenesis. Research conducted by Kang et al. (2011) stated that 2% PRF extract can increase the proliferation of human alveolar bone marrow stem cells (hABMSCs) (16). In this study, the treatment group was given PRF with a concentration of 2%.

Similar to other mesenchymal stem cells, various studies have shown that AMSCs have the potential to undergo adipogenesis, chondrogenesis, osteogenesis, myogenesis, and vasculogenesis. Where are the various reagents from the previous study could induce AMSCs differentiation into cardiomyocyte-like cells including 5-azacytidine (5-Aza), angiotensin II (Ang II), and transforming growth factor- β1 (TGF- β 1). In a study conducted by Safwani et al, in 2011 it was stated that 5-Aza was not effective for inducing cardio genesis in AMSCs. Therefore, in this study, it is suggested that the addition of growth factors is believed to help the induction of AMSCs. A study conducted by Planat Barnard et al, in 2004 confirmed that the use of growth factors for cardiomyocyte-like cells differentiation is necessary (17).

The role of platelets in cardiomyocyte-like cells differentiation is said to lie in the growth factors contained therein. Where platelet activation that occurs in the cardiac recovery phase after ischemic damage causes the release of the alpha-granule component which is the main content of platelets which will mediate the formation of cardiac progenitor cells to become mature cardiomyocyte-like cells (18).

Conclusion

There is a beneficial effect of the addition of injectable platelet-rich fibrin to accelerate differentiation of adipose-derived mesenchymal stem cells into cardiomyocyte-like cells. It can be seen by increasing the expression of GATA-4 and Troponin. However, further

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research is needed by examining markers at each stage of cardiomyocyte formation starting from cardiac mesoderm to mature cardiomyocyte-like cells.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ABBREVIATION

AMSCs	:	Adipose-derived Mesenchymal Stem Cells
ATP	:	Adenosine Triphosphate
BAX	:	BCL-2-associated X protein
BCL2	:	B-Cell Lymphoma 2
BMSCs	:	Bone marrow-derived Mesenchymal Stem Cells
CD44	:	Cluster of Differentiation 44
CLCs	:	cardiomyocyte-like cells
cTnT	:	cardiac Troponin-T
EGF	:	epidermal growth factor
ITD	:	Institute of Tropical Diseases (Universitas Airlangga)
IGF-1	:	insulin like growth factor-1
PRF	:	Platelet-rich Fibrin
PRP	:	Platelet-rich Plasma
ROS	:	Reactive oxygen species
SCF	:	Stem Cell Factor
SLF	:	Steel Factor
SPSS	:	Statistical Package for Social Sciences
TGF-β	:	Transforming Growth Factor- β
VEGF	:	Vascular Endothelial Growth Factor

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