ORIGINAL ARTICLE

Association between donor's age and expression of cardiomyocyte marker quantity on adipocyte-derived mesenchymal stem cell

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ABSTRACT

Aim Adipocyte-derived mesenchymal stem cells (AMSCs) have recently been studied as a potential new approach for regeneration treatment of heart failure and ischemic myocardium. However, donor's age is thought to affect the ability of AMSC cells to differentiate into cardiomyocytes. This study aimed to analyse the association between donor's age with the expression of cardiomyocyte marker cardiac isoform of Troponin-T (cTnT) quantity on AMSCs.

Methods Subcutaneous adipose tissue was taken from donors of 3 different age groups (30-40, 41-50, and >50 years old), and then processed into AMSC cells by insert culture. AMSC cell phenotype was identified by assessing the quantity of mesenchymal markers expression (CD34, CD45, CD90, CD105) using indirect immunofluorescence technique. Afterwards, cTnT expression, a marker for cardiomyocyte differentiation from AMSC cells, was evaluated under the fluorescent microscope. The comparison was done between the 3 different age groups.

Results The comparative test and post hoc analysis showed that there were differences in the expression of CD34, CD45, CD90, CD105, and cTnT between the three age groups (all $p<0.05$). Furthermore, there were strong negative correlations between age and CD34, CD90, CD105 and cTnT ($r= -0.844, -0.914, -0.899$, and -0.738, respectively), while the correlation was positive between age and CD45 ($r=0.899$).

Conclusion Expression quantity of mesenchymal markers and cardiomyocyte markers from AMSC cells were affected by the donor's age. The higher the donor age group, the lower the expression quantity of mesenchymal markers (except CD45) and cTnT.

Key words: cardiomyocyte, donor, mesenchymal stem cells, troponin T

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Original submission:
22 February 2022;
Revised submission:
14 April 2022;
Accepted:
02 June 2022
doi: 10.17392/1478-22

Med Glas (Zenica) 2022; 19(2): 120-126

INTRODUCTION

Coronary heart disease (CHD) is one of the primary worldwide causes of mortality and morbidity, including heart failure. Yet, available heart failure therapy nowadays is more aimed at maintaining the remaining heart function rather than restoring the function completely (1), thus, a new approach is needed in dealing with heart failure that is able to produce new contractile tissue or restore ischemia in the myocardium. The identification of a variety of stem cells with varying degrees of flexibility has paved the way for the regeneration of injured myocardium and restoration of cardiac function (2).

Stem cells are a source of immature regenerative cells that can differentiate into a variety of cell types, making them ideal for tissue regeneration, however, the efficiency of differentiation varies based on the type of cell as well as the methodology used (3). There are two types of stem cells, namely adult stem cells (ASCs) and embryonic stem cells (ESCs). However, ESCs have ethical constraints in their acquisition process, therefore, currently the use of ASCs is increasingly being investigated to replace the use of ESCs. One type of ASCs that has been widely studied are various types of mesenchymal stem cells (MSCs), such as bone-marrow derived mesenchymal stem cells (BM-MSC), adipocyte-derived mesenchymal stem cells (AMSC), and hematopoietic stem cells (HSC) (4).

Adipose tissue, which contains mature adipocytes as well as AMSCs, has recently been identified as a potential source of progenitor cells (5). Compared to other tissues, adipose tissue has several advantages as stem cell precursors, such as high availability, easier harvest access through subcutaneous lipo-aspiration, and lesser ethical controversy (because cells are obtained autologously, unlike ESCs) (6). The capacity of AMSC cells to expand in vitro is also superior to that of BM-MSC in terms of required time and cell viability in long-term storage (7).

Nevertheless, published literature of the utilization of AMSC for cardiomyocyte cell differentiation cells is relatively few and falls behind compared to more widely published studies of BM-MSCs utilization (8, 9). In 2004 AMSCs were found for the first time to spontaneously differentiate into pulsing cardiomyocytes, although with poor differentiation (10). This poor differentiation occurs because of the heterogeneity of the population that composes AMSCs. In addition, many properties of stem cells can be affected by factors such as aging and metabolic disorders (11). Several studies have shown that MSCs isolated from elderly individuals exhibit reduced proliferative activity, osteogenic potential and cell viability, compared to younger donors (11,12). With the aging population, cellular treatment will become more relevant in the future for this subject group, which is the primary target population for such therapy (13).

However, to the best of our knowledge, studies about the effect of donor age on the ability of AMSC cells to differentiate into cardiomyocytes are very limited (14, 15). Therefore, it is important to look at donor's age as a factor in determining whether cellular therapy, particularly for the treatment of coronary heart disease, may achieve the desired results in people employing autologous stem cells.

The aim of this study was to investigate the association between donor's age with the expression of cardiomyocyte marker cardiac isoform of troponin-T (cTnT) quantity on AMSCs.

MATERIALS AND METHODS

Study design and population

Research was carried out at the Centre for Research and Development of Stem Cells, Institute of Tropical Disease (ITD), Universitas Airlangga from July to October 2021. A sample was AMSC passage 4 cells cultured from abdominal subcutaneous adipose tissue of healthy human donors with normal blood profiles, no history of diabetes, hypertension, CHD, and no obesity from 3 different age groups (30-40 years old, 41-50 and above 50 years of age), where each age group was represented by one human subject. The sample size replication in each group was 8-sample replication, calculated using Federer method (16).

Subcutaneous adipose tissue was obtained by abdominoplasty, and then processed into AMSC cells according to the insert culture protocol by Jumabay et al. (17). Furthermore, these AMSC passage 4 cells were identified for their cell phenotype by assessing quantity of the expression of mesenchymal markers, namely CD90+, CD105+, CD34-, and CD45-, using indirect

immunofluorescence. AMSC cells were then cultured on a special media for cardiomyocyte differentiation to see the expression quantity of cardiomyocyte markers (cTnT) as a marker of cardiomyocyte existence (Figure 1).

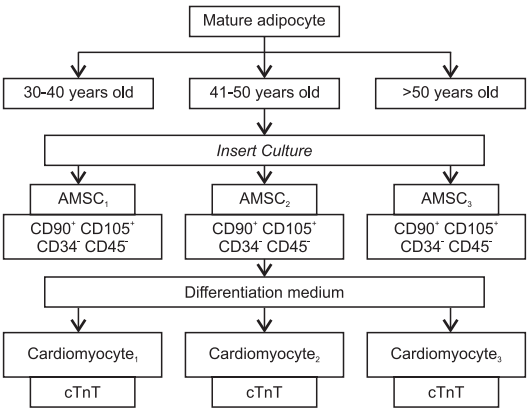

Figure 1. Study design flowchart AMSC, adipocyte-derived mesenchymal stem cells

Our study protocol was approved by the Institutional Ethics Committee of Dr. Soetomo General Hospital (274/EC/KEPK/FKUA/2021). An informed consent for adipose tissue sampling procedures and participation in research studies was obtained from all patients. All data that could reveal the identity of the subjects have been omitted.

Methods

Anatomical specimen collection. Mature adipocyte tissue was obtained from loose subcutaneous tissue by abdominoplasty procedure with an estimated weight of 10 grams stored in 50 mL conical tube and on ice at -70 °C and taken in plastic containers to the laboratory. Next, 1-2 grams of adipocyte tissue were washed with 5 mL of phosphate buffer saline (PBS) room temperature in a 50 mL tube and the sample was placed in a 10 cm plastic dish.

Isolation and culture of AMSC cells from adipocytes. The adipose tissue was then transferred to an Erlenmeyer tube and added with 10 mL of collagenase type 1 (Worthington, USA) solution (1:1 ratio), while incubating for 45 minutes at 37 □. Then a stopper (1 mL of PBS) was added to the Erlenmeyer tube for further incubation for 15 minutes, and the tissue was then filtered into a glass beaker. The supernatant was then collected in Corning tube and centrifuged at 3000 rpm for 5 minutes. The supernatant was then discarded, the remaining pellet was washed with PBS one time and the process was repeated once more, and then the precipitate obtained was washed with PBS and then put into α-MEM culture medium (Gibco, USA) in three different conical tubes with an estimated volume of 10 mL each. The pellet cells in the culture medium were then filtered with a 100 **µ** and then 70 **µ** cell strainer. After that, the pellet cells were placed in a petri dish and incubated in a CO_2 incubator.

Identification of expression of CD90, CD105, CD34 and CD45. Identification of cell phenotypes was carried out by assessing the expression of markers of mesenchymal stem cells through the immunocytochemical method (immunofluorescence indirect). Each AMSC cell was harvested, put into a 15 mL plastic tube, fixed with methanol, and after 15 minutes given anti-CD90, anti-CD45, anti-CD34 and anti-CD45 reagents, then washed with PBS solution, and after that evaluated using particle label fluorochrome Alexa Fluor 488 and observed under fluorescence microscope with a 100x magnification (Automated fluorescence microscope, BX63, Olympus, USA).

Induction of AMSC cells on cardiomyocyte differentiation media. Good quality AMSC cells were then cultured on special media for cardiomyocyte differentiation, namely StemCelldiff (Stemcell, USA). The differentiation medium consists of 3 parts, differentiation medium A, differentiation medium B, and maintenance medium. The differentiation process was carried out according to the protocol attached to the medium worksheet.

Assessment of cardiomyocyte surface marker expression

An assessment of cTnT expression in AMSC cell culture results to detect the presence of cardiomyocytes was carried out on day 21 after AMSC cells were exposed to a differentiation medium. The differentiation medium was changed every 5-7 days according to the protocol until the final target day of observation was reached. The monolayer cells that had been incubated were fixed using 3% formalin buffer, then the object glass was washed and blocked using PBS for 15 minutes. Next, immunocytochemical examination with secondary staining of 10 µl according to the label, was intended to see the expression of

cTnT. The staining process was carried out in a dark room, then the preparations were incubated for 1 hour at 37 °C. The results were read using a fluorescent microscope by assessing the presence of antibody and antigen bonds both on the surface and inside the cells.

Statistical analysis

Continuous data were presented as mean ± SD. For data distribution normality test, Saphiro Wilk test was used. Analyses to evaluate the difference in the quantity of mesenchymal marker expression and cardiomyocyte marker expression (cTnT) based on age group were done using ANOVA comparison test continued by Tukey post-hoc analysis for normally distributed data, and Kruskal-Walli's comparison test continued by Mann-Whitney post hoc test for skewed data. Analyses to measure the correlation between donor age and the quantity of expression of cardiomyocyte markers in AMSC cells were done using Pearson or Spearman test analysis, depending on the data distribution. A $p<0.05$ was considered statistically significant.

RESULTS

Mature adipocyte tissue was obtained from 3 male human donors aged 33 years, 48 years, and 58 years (for representation of age group 30-40, 41-50, and >50 years, respectively). The BMI of the donor aged 33 years was 25.5 kg/m^2; of the donor aged 38 years old it was 26.1 kg/m^2; and of the donor aged 58 years old it was 26.5 kg/m^2. All donors were healthy without any comorbidities.

Characterization of the human-mesenchymal stem cell phenotype was carried out successfully on AMSC passage 4 cells by identifying the phenotypes of CD90+, CD105+, CD34-, and CD45- as surface markers identified using indirect immunofluorescence (Figure 2).

The quantitative data of mesenchymal marker expression were then grouped by age, with each group consisting of 8 replication samples. The highest mean of CD34, CD90, and CD105 expression was in the 30-40 years age group, 6.95±0.72, 13.09±0.29, and 17.94±0.45, respectively, and the lowest in >50 years age group, 4.19±0.33, 9.53±0.51, and 12.73±0.47, respectively. In the contrary, the highest mean of CD45 expression was in the >50 years age group, 5.09±0.25, while the lowest was in 30-40 years age group, 2.22±0.32 (Table 1).

The expression quantity of cardiomyocyte markers (cTnT) from the age group of 30-40 years old was 8.88±0.54, from the age group of 41-50 years old was 7.92±0.61, and from the age group of >50 years old was 7.33±0.68 (Figure 3).

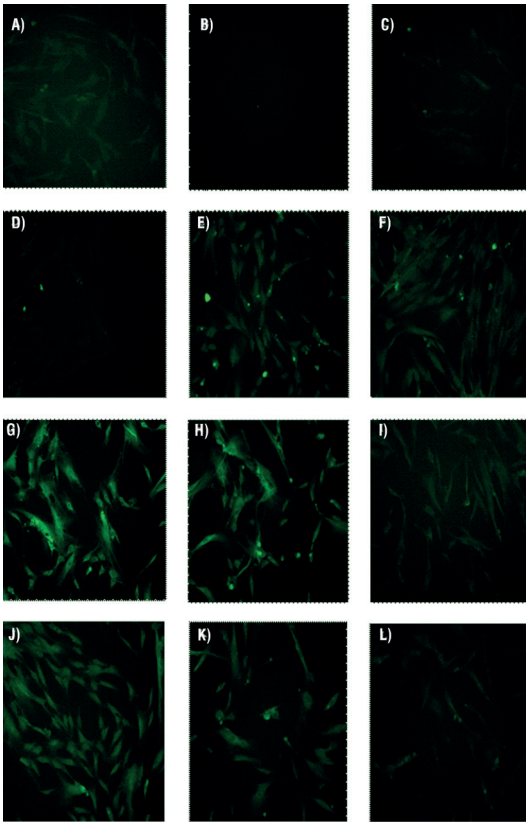

Figure 2. Expression of immunocytochemistry staining in adipocyte-derived mesenchymal stem cells (AMSC). CD34- expression taken in patients aged A) 33 years, B) 48 years, and C) 58 years; CD45- expression taken in patients aged D) 33 years, E) 48 years, and F) 58 years; CD90+ expression taken in patients aged G) 33 years, H) 48 years, and I) 58 years; CD105+ expression taken in patients aged J) 33 years, K) 48 years, and L) 58 years (L)

Table 1. Calculation results of mean and standard deviation (SD) mesenchymal markers by age group

Age group	N	CD34		CD45		CD90		CD105	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
30 – 40 years	8	6.950	0.721	2.223	0.323	13.090	0.288	17.941	0.457
41 – 50 years	8	6.190	0.509	2.775	0.183	11.139	0.811	17.156	0.282
> 50 years	8	4.198	0.332	5.084	0.252	9.533	0.510	12.728	0.472

N, number of replications of adipocyte-derived mesenchymal stem cells

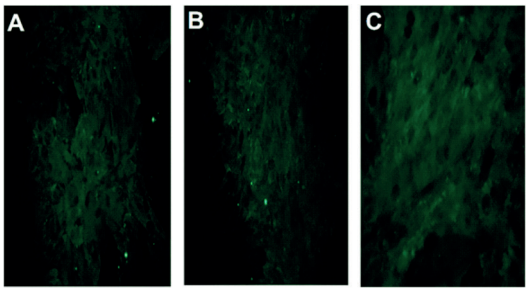

Figure 3. Overview of cardiac isoform of Troponin-T expression taken in patients aged A) 33 years, B) 48 years, and C) 58 years

CD34 and cTnT expressions were normally distributed (Saphiro Wilk p=0.09 and p=0.88, respectively), so ANOVA and Tukey post hoc analysis were used. Meanwhile the expression data for CD45, CD90, and CD105 were not normally distributed (Saphiro Wilk p=0.00, p=0.02, p=0.00, respectively), therefore, Kruskal Wallis test and Mann Whitney post hoc analysis were used (Table 2). The results of the ANOVA and Kruskal Wallis tests showed that there were differences in the expression of CD34, CD45, CD90, CD105, and cTnT between the age groups (all p<0.05).

Further post-hoc analysis using Tukey and Mann Whitney tests showed that there was significant difference between every age group category on CD34, CD45, CD90, CD105, and cTnT expression (all p<0.05) (Table 2).

Table 2. Mesenchymal marker expression difference test results and cardiomyocytes by age group

Variable	ANOVA/ Kruskal Wallis p	Interpretation	Post hoc analysis		p
CD34	0,000	Significant difference	30 – 40 years	40 – 50 years	0.028
			30 – 40 years	> 50 years	0.000
			40 – 50 years	> 50 years	0.000
CD45	0,000	Significant difference	30 – 40 years	40 – 50 years	0.006
			30 – 40 years	> 50 years	0.001
			40 – 50 years	> 50 years	0.001
CD90	0,000	Significant difference	30 – 40 years	40 – 50 years	0.002
			30 – 40 years	> 50 years	0.001
			40 – 50 years	> 50 years	0.002
CD105	0,000	Significant difference	30 – 40 years	40 – 50 years	0.006
			30 – 40 years	> 50 years	0.001
			40 - 50 years	> 50 years	0.001
cTnT	0,000	Significant difference	30 – 40 years	40 – 50 years	0.028
			30 – 40 years	> 50 years	0.000
			40 – 50 years	> 50 years	0.000

For secondary analysis, the results of the Spearman correlation test showed that the expression of CD34, CD45, CD90, CD105 and cTnT was strongly correlated with thet age groups (R= -0.844, p<0.001; R=0.899, p<0.001; R= -0.914, p<0.001; R= -0.899, p<0.001; and R= -0.738, p<0.001, respectively). The correlation showed inverse relation, which meanta the higher age group, the lower the quantity of CD34, CD90, CD105 and cTnT expression. Meanwhile for CD45 expression, there was a positive correlation meaning that the higher the age group, the greater the quantity of CD45 expression.

DISCUSSION

In this study, we found that the expression quantity of the mesenchymal markers CD34, CD90, and CD105 was highest in the 30-40 years age group, and the lowest expression quantity in the >50-year age group. Similar results were obtained in observing cardiac biomarkers (cTnT). The highest quantity of cTnT expression was in the 30-40 years age group, while the lowest was in the >50-year age group. However, different results were obtained in the calculation of CD45. The highest quantity of CD45 was found in the age group > 50 years, and the lowest quantity in the age group 30-40 years. These results are not in accordance with the theory that elder individuals exhibit more reduced proliferative activity (11), and are not in line with the findings in the calculation of other mesenchymal markers and cardiomyocyte markers in our study. High count results or false positives can result from the calculation of the luminescence of artifacts in the microscope's field of view. While low or false negative results can be caused by failure in cell staining, uneven distribution of cells, and the number of positions of cells that accumulate in the microscope field of view.

Furthermore, from the statistical analysis result, our study showed that there were significant differences in the quantity of expression of mesenchymal markers CD34, CD45, CD90, CD105, and expression of cardiomyocyte markers (cTnT) between the age groups. The secondary analysis also showed that the expression of CD34, CD45, CD90, CD105 and cTnT correlated with the age groups. The highest correlation was in the age group with CD90 expression. These results indicate that the higher the age group, the lower the expression quantity of CD34, CD90, CD105 and cTnT. Only CD45 expression quantity showed a positive correlation with increasing donor's age in our study.

Overall, our results support previous research conducted by Choudhery et al. (15), where they attempted to study the growth characteristics and

in vitro regenerative potential of human adipose tissue-derived mesenchymal stem cells (hAT-MSCs) obtained from donors of various age groups (young, adult and old) in combination with gene expression profile, superoxide dismutase activity and senescence level. Their result showed that the number, frequency, and population of MSCs will decrease significantly as the senescence level increases with increasing donor age. The diminished functional capacity of stem cells is thought to be linked to decreased organ repair capabilities as organisms age. It is thought that cells in the older age have undergone age-related alterations, which has contributed to tissue rejuvenation's diminished potential (15). Furthermore, age-related illnesses such as diabetes and heart failure impair the function of endogenous progenitor cells (12).

The success of cell-based therapy can be influenced by donor age factors related to the potential for cell expansion and differentiation (18), and our primary results study also support this statement, because it was found that the higher the age group, the lower the differentiation expression quantity of cardiomyocyte markers (cTnT). A previous study has shown that age decreased the frequency of stem cells represented by colony-forming unit fibroblast assay, and also decreased total stromal vascular fraction gain and MSCs proliferation rate although not significantly (19). The aging process is thought to increase cellular senescence so that the ability of MSCs to undergo adipogenic and osteogenic differentiation will decrease (20). Further studies are needed to investigate the presence of factors or treatments that can increase and optimize the expression of cardiomyocyte markers in elderly stem cell donors.

In conclusion, our study is the first to show significant differences in the expression quantity of mesenchymal markers (CD34, CD45, CD90, CD105) and cardiomyocyte markers (cTnT) from AMSC cells obtained from donors of different age groups. The higher the donor age group, the lower the expression quantity of mesenchymal markers (except CD45) and cardiomyocyte markers (cTnT). Future studies should take donor's age variable into consideration prior to samples collection.

FUNDING

No specific funding was received for this study.

TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

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