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Association between reversine dose and increased plasticity of dedifferentiated fat (DFAT cells) into cardiac derived cells

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SOUHRN

Cíl: Analyzovat souvislost mezi podáním reversinu a zvýšenou plasticitou buněk DFAT schopných dělení na různé typy buněk.
Metoda: Vykultivované buňky DFAT byly rozděleny do čtyř skupin podle dávky reversinu: na kontrolní (bez reversinu) skupinu a na skupiny s aplikací reversinu v dávkách 10 nM, 20 nM a 40 nM. Každá skupina prochází několika stadii vývoje před další diferenciací na kardiomyocyty (identifikované expresí cTnT), buňky hladké svaloviny (vascular smooth muscle cells, VSMC) (označené expresí afta-SMA) a buňky cévního endotelu (identifikované expresí CD31).
Výsledek: V každé skupině buněk DFAT s aplikací reversinu byly nalezeny statisticky významné rozdíly v expresi cTnT, alfa-SMA a CD31 (p = 0,003; resp. < 0,001 a < 0,001). *Post hoc* analýza s použitím Tukeyova testu prokázala, že pouze ve skupině s reversinem v dávce 10 nM došlo ke statisticky významnému rozdílu oproti kontrolní skupině (p = 0,002) v expresi cTnT a ve skupinách s reversinem v dávkách 10 nM a 20 nM k rozdílu v expresi alfa-SMA a CD31 (p = 0,028, resp. p < 0,001).
Závěry: Tato studie prokázala vztah mezi dávkou reversinu a zvýšenou plasticitou buněk DFAT schopných diferenciace na kardiomyocyty (cTnT), VSMC (alfa-SMA) a buňky cévního endotelu (CD31).

Keywords:
Cardiomyocyte
DFAT
Reversine
Vascular endothelial cells
VSMCs

ABSTRACT

Aim: To analyze the association between reversine and increased plasticity of DFAT into cardiac derivative cells.
Method: The cultured DFAT cells were divided into four groups based on reversine dose: control (no reversine), 10 nM, 20 nM, and 40 nM reversine. Each group will go through several stages of passage before further differentiation into cardiomyocytes (marked by cTnT expression), VSCMs (marked by alpha-SMA expression), and vascular endothelial cells (marked by alpha-SMA expression) (marked by CD31 expression).
Result: There were significant differences in the expression of cTnT, alpha-SMA, and CD31 (p = 0.003, p <0.001, and p <0.001, respectively) in each group of DFAT cells that received reversine. From post-hoc analysis with Tukey test, it was found that only the 10 nM reversine group produced a significant difference compared to the control group (p = 0.002) for cTnT expression and reversine 10 nM and 20 nM group for α-SMA expression and CD31 expression (p = 0.028 and p <0.001, respectively).
Conclusions: This study proves that there is a relationship between reversine and increased plasticity of DFAT cells into cardiac derived cells in the form of cardiomyocytes (cTnT), VSMCs (alpha-SMA), and vascular endothelial cells (CD31).

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Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Between 2010 and 2030, the prevalence rate is expected to climb by 10%.[1] CVD is also the major cause of morbidity and mortality in Indonesia, accounting for one third of all deaths,[2] and estimated to cause more than 470 000 deaths annually.[3] Advances in therapeutic modalities for patients after acute myocardial infarction (AMI) have led to a reduction in early mortality. Despite that, this approach still cannot prevent interstitial, vascular, and cardiac cell death. Recently, there has been a new understanding that regenerative processes can also occur in the myocardium. The use of stem cells has begun to be developed as a promising therapeutic approach with high expectations to regenerate the function of the infarcted myocardium and repair injured endothelial cells.[4–7] Besides embryonic stem cells (ESCs), adult stem cells (ASCs) are one of the two multipotent stem cells, that can be obtained from many organs.[8] Adipose tissue (AT) is an ASC that contains more stem cells than other tissues and may be obtained through a less invasive surgical process.[9] Previous in vitro experiments have revealed that mature AT can be converted into multipotent stem cells.[10] The "ceiling culture" approach is used to create adipocyte-derived cell lines, also known as dedifferentiated fat cells (DFAT). DFAT cells have a fibroblast-like appearance and possess the properties of multilineage precursor cells.[11] It has been shown that DFAT cells can differentiate into some important cardiovascular component, such as vascular smooth muscle cells (VSMCs), identified by smoothelin and alpha-SMA expression,[12] vascular endothelial cell (EC) marked by CD31 expression, and cardiomyoctye (CM) marked by cardiac troponin T (cTnT) in immunocytochemical analysis.[13] All of the components are necessary for circulatory regeneration and myocardial contractility improvement.[14] Reversine is a 2,6-disubstituted purine derivative which not only inhibits the human adenosine A3 receptor, non-muscle myosin II heavy chain, mitogen activated protein kinase-1 (MEK1), and Aurora B kinase, but also induces the differentiation of myogenic competence cells into multipotent mesenchymal progenitor cells and the differentiation of mouse macrophages into mesenchymal progenitor-like cells.[15,16] Currently, there is no research concerning the effect of reversine on increasing the plasticity of DFAT cells into cardiac derived cells in the form of VSMCs, CM, and EC. Our study aimed to analyze the association between reversine and increased plasticity of DFAT into cardiac derivative cells.

Methodology

This study is an exploratory experimental study (in vitro) with a cross-sectional design that aims to analyze whether the administration of reversine will increase the plasticity of DFAT cells, which differentiate into vascular EC, CM, and VSMCs characterized by the expression of CD31, cTnT, and Alpha-SMA markers, respectively. All information that may disclose the subjects' identities has been removed. This study's sample population was DFAT cells cultured from abdominal subcutaneous adipose tissue of a healthy human subject. The DFAT cells are then separated into four groups where each group will be added with experimental variables: control (without reversine), 10 nM reversine, 20 nM reversine, and 40 nM reversine. The sample size in each group was eight samples DFAT cells replication, calculated using Higgins and Klinbaum formula.[17] The study outcome was quantitative differentiation of cardiomyocytes (marked by cTnT expression), VSCMs (marked by alpha-SMA expression), and vascular endothelial cells (marked by CD31 expression) from each reversine dose group compared to control group.

Statement of ethics

The research is carried out at the Center for Research and Development of Stem Cells, Institute of Tropical Diseases (ITD), Universitas Airlangga from July 2021 to March 2022. The Institutional Ethics Committee of Dr. Soetomo General Hospital approved our study protocol (53/EC/KEPK/FKUA/2022). Informed consent for adipose tissue sampling procedures and participation in research studies was obtained from all patients.

Sample collection and culture isolation

For sample collection, mature adipocyte tissue was obtained from loose subcutaneous tissue through a laparotomy procedure with an estimated weight of 10 grams, stored in 50 mL conical tube and ice at –70 degree of Celcius. Then 1–2 grams of adipocyte tissue was washed with 5 mL PBS once in a 50 ml tube. The next step is DFAT cell culture and adipocyte isolation with insert culture.[18] One gram of mature adipocyte tissue was mashed, and 0.1% collagenase type I solution was added, then filtered through a nylon filter (100 µm) and centrifuged for 3 minutes. Then the supernatant was taken, rinsed three times with αMEM solution. A total of 30–50 µL of adipocytes obtained from the creamy top layer were then transferred into six well plates filtered with 70 µL and incubated for five days in a culture medium. The DFAT cell will sink to the bottom of the dish and be picked up. It takes 5×10^4 adipocytes (less than 100 mg adipose tissue) to obtain an adequate quantity of DFAT cells. The expression of mesenchymal stem cells markers, such as CD90+, CD105+, CD34–, and CD45–, was assessed using an immunocytochemical method (immunofluorescence indirect) to determine cell morphologies. DFAT cells were harvested, fixed using methanol, and after 15 minutes was added with anti-CD90, anti-CD45, anti-CD34 and anti-CD45 reagents. Then the mixture were washed with PBS solution, dropped on object glass, and then evaluated using fluorescence microscopy. Furtherore, DFAT cells were induced on differentiation media of VSMCs, CM, and EC. Good quality DFAT cells were cultured on special media for differentiation of VSMCs, namely alpha-MEM, differentiation of CM, namely StemCelldiff (Stemcell, USA), and differentiation of EC, namely CSTI-303MSC (Cell Science & Technology Institute, Miyagi, Japan). Each differentiation medium consists of 3 parts: differentiation medium A, differentiation medium B, and maintenance medium. The last stage was the assessment of the expression of α-SMA (marker for VSMCs), cTnT (marker for CM), and CD31 (marker for EC). Assessment of the expression of each marker in ADSC/DFAT culture results was carried out after ADSC and DFAT cells were exposed to differentiation

medium on days 7 and 21 for α-SMA expression, day 7, 14, and 21 for cTnT expression, and days 6 and 8 for CD31 expression. The differentiation medium was changed daily according to the protocol until the final target day of observation was reached. A fluorescent microscope was used to examine the presence of antibody and antigen binding on the cell's surface and inside.

Statistical analysis

The SPSS 25.0 software was used to analyze the data (IBM Corp., Armonk, New York). The mean SD was used to present continuous data. We used the Saphiro Wilk test to determine the data distribution normality. The primary goal of this study is to see if there is a difference in the number of cardiomyocytes (marked by cTnT expression), VSCMs (marked by alpha-SMA expression), and vascular endothelial cells (marked by CD31 expression) from each reversine dose group compared to the control group, using an ANOVA comparison test followed by Tukey post--hoc analysis for normally distributed data, and a Kruskal--Wallis comparison test followed by Mann-Whitney for undistributed data.

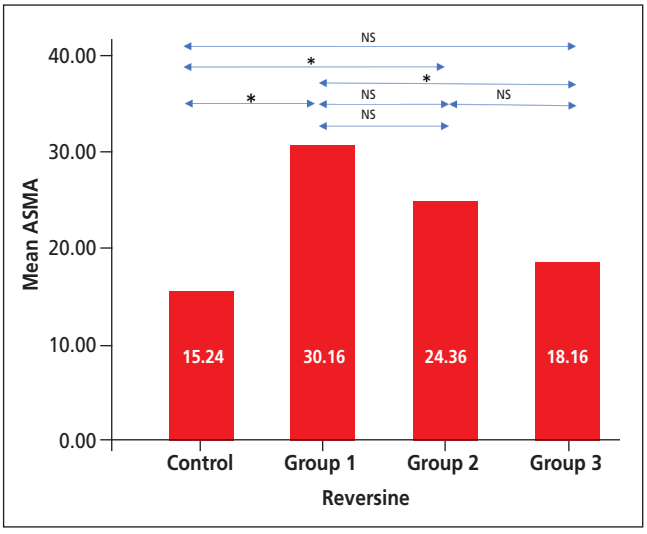

Fig. 1 – Differences in the mean expression of the VSMC marker (α-SMA [ASMA]) based on the reversine dose group (control group, group 1 (10 nM), group 2 (20 nM), group 3 (40 nM). *P* value <0.05 (*) was considered statistically significant, NS – non significant.

Results

Descriptive result

The sample donor characteristic was a healthy 32-year--old human female donors with normal blood profiles, no history of diabetes, hypertension, CAD, malignancy, and were not obese. Subcutaneous abdominoplasty was used to harvest the adipose tissue and further proccessed. The following is a descriptive calculation of the expression variables for VSMCs, CM, and EC markers in the control group and the reversine group, namely reversine 10 nM, reversine 20 nM, and reversine 40 nM (Table 1).

From Table 1, it can be seen that the highest average quantity of cTnT and alpha-SMA expression in group 1 (reversine 10 nM) was 9.00 ± 1.03 and 30.18 ± 8.22, respectively, while the lowest was in the control group, with 7.04 ± 1.07 and 15.23 ± 3.67, respectively. Meanwhile, the highest CD31 expression quantity was found in group 2 (reversine 20 nM), with 41.35 ± 10.96, and the lowest was in the control group 11.23 ± 7.08. Normality test was carried out using the Shapiro-Wilk test. All three variables has a normal distribution. (p = 0.134, 0.062, 0.083, respectively).

Effect of reversine dose on alpha-SMA, cTnT, and CD31 expression of DFAT cells

For VSMCs differentiation, ANOVA analysis showed that there are significant differences in the expression of α-SMA in each group of DFAT cells receiving various doses of reversin (p < 0.001) (Table 2).

From the Tukey test which is a post-hoc analysis of the ANOVA test, it was found that only the reversine dose 10 nM group and the reversine dose 20 nM group resulted in a significant increase in α-SMA expression compared to the control group (30.18 ± 8.22 vs. 15.23 ± 3.67; p = 0.028) and (24,35 ± 2.80 vs. 15.23 ± 3.67; p <0.001), respectively. It was also found that α-SMA expression was significantly lower in Reversine 40 nM group compared to Reversine 10 nM group (p <0.001) (Fig. 1).

For CM differentiation, from the Table 2, it was known from ANOVA analysis that there are significant differences in cTnT expression in each group of DFAT cells receiving various doses of reversine (p <0.001). From Tukey pot-hoc analysis, significant difference was only found between the reversine 10 nM group and control group (9.00 ± 1.03 vs. 7.05 ± 1.07; p = 0.002). Meanwhile, no statistical difference was found between other groups, however, the data showed the trend of increasing cTnT expression from reversine 10 nM group to reversine 20 nM group, and then decreasing in 40 nM group (Fig. 2).

For EC differentiation, it was found that there was a significant difference of CD31 expression in each group of DFAT cells that received various doses of reversine. From the Tukey post-hoc analysis, it was found that the Reversine dose 10 nM group and the Reversine dose 20

Table 1 – Descriptive result of cardiac derived cells marker by reversine dose group

Variable	N (replication)	Total		Control		Group 1 Reversine 10 nM		Group 2 Reversine 20 nM		Group 3 Reversine 40 nM	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
cTnT	8	7.99	1.16	7.04	1.07	9.00	1.03	8.17	0.55	7.74	1.10
α-SMA	8	21.98	7.48	15.23	3.67	30.18	8.22	24.35	2.80	18.16	2.65
CD31	8	24.57	13.96	11.23	7.08	24.66	10.19	41.35	10.96	16.12	5.06

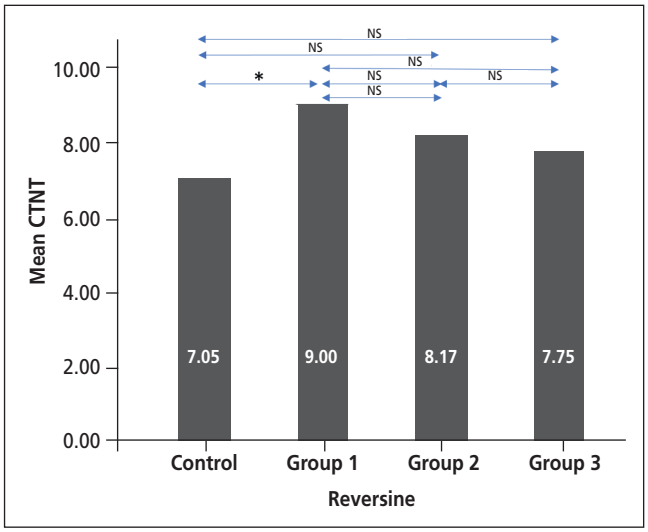

Fig. 2. Differences in the mean expression of the cardiomyocyte marker (cTnT) based on the reversine dose group (control group, group 1 [10 nM], group 2 [20 nM], group 3 [40 nM]). *P* value <0.05 (*) was considered statistically significant. NS – non significant.

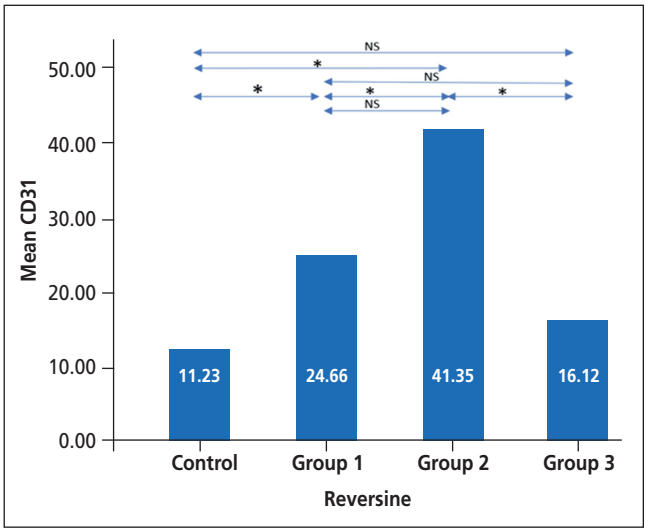

Fig. 3 – Differences in the mean expression of the vascular endothelial cell marker (CD31) based on the reversine dose group (control group, group 1 [10 nM], group 2 [20 nM], group 3 [40 nM]). *P* value <0.05 (*) was considered statistically significant, NS – non significant.

nM group resulted in a significant increase in CD31 expression compared to the control group (24.66 ± 10.19 vs 11.23 ± 7.08; $p = 0.028$) and (41.35 ± 10.96 vs. 11.23 ± 7.08; $p < 0.001$), respectively. There was also significant increase in CD31 expression from reversine 1 0nM group to reversine 20 nM group ($p = 0.01$), and then decrease CD31 expression from reversine 20 nM group to reversine 40 nM group (p<0.001) (Fig. 3).

Discussion

The advantages of DFAT over ASCs that they were detected earlier (± on 5th day) with a larger number of cells, easier access, and isolation method, homogeneity, and lower immunogenicity after transplantation.[19,20] Previous research has demonstrated that DFAT cells may transdifferentiate into chondrocytes, osteoblasts, smooth muscle cells, ske-

Table 2 – Difference in expression of VSMC markers (α-SMA), cardiomyocyte markers (cTnT), and EC markers (CD31) based on reversine dosage group

Variable	ANOVA *p*-value	Description	Post-hoc test		Tukey *p*-value	Significant difference
α-SMA	0.000	There is a significant difference between groups	Control	Group 1 (10 nM)	0.028	Yes
			Control	Group 2 (20 nM)	0.000	Yes
			Control	Group 3 (40 nM)	0.279	No
			Group 1 (10 nM)	Group 2 (20 nM)	0.109	No
			Group 2 (20 nM)	Group 3 (40 nM)	0.105	No
			Group 1 (10 nM)	Group 3 (40 nM)	0.000	Yes
cTnT	0.000	There is a significant difference between groups	Control	Group 1 (10 nM)	0.002	Yes
			Control	Group 2 (20 nM)	0.077	No
			Control	Group 3 (40 nM)	0.279	No
			Group 1 (10 nM)	Group 2 (20 nM)	0.092	No
			Group 2 (20 nM)	Group 3 (40 nM)	0.105	No
			Group 1 (10 nM)	Group 3 (40 nM)	0.253	No
CD31	0.000	There is a significant difference between groups	Control	Group 1 (10 nM)	0.028	Yes
			Control	Group 2 (20 nM)	0.000	Yes
			Control	Group 3 (40 nM)	0.279	No
			Group 1 (10 nM)	Group 2 (20 nM)	0.010	Yes
			Group 2 (20 nM)	Group 3 (40 nM)	0.000	Yes
			Group 1 (10 nM)	Group 3 (40 nM)	0.105	No

letal myocytes, cardiomyocytes, vascular smooth muscle cells, and vascular endothelial cells in vitro or in vivo given the right growing conditions.[11] Similar as our study, previous study also indicated the use of specific marker of α-SMA for VSMCs, cTNT for CM, and CD31 for EC.[12,13] The use of cardiac derived cells from DFAT is thought to have potential benefit for treatment for cardiovacular disease, for example, treatment with VSMCs and their progenitors has been shown to induce neovascularization, wound healing, and improve myocardial contractility.[14] In another study, transplanted DFAT cells aggregated in the infarcted myocardium at 8 weeks following cell transplantation, produced cardiac actin sarcomer, and significantly (*p* 0.05) enhanced capillary density in the infarcted region as compared to the hearts of the injected salt control mice.[21]

However, no prior research has looked at the possibility of increasing the plasticity of DFAT differentiation into cardiac-derived cells. Our finding was the first that prove that reversine can increase the plasticity of human DFAT cells into cardiac derived cells in the form of cardiomyocytes (cTnT), VSMCs (alpha-SMA), and vascular endothelial cells (CD31). Previous studies have only shown that reversine have the capacity to induce differentiation in other tissue. Reversine, an adenosine A3 receptor antagonist, has been shown to stimulate the differentiation of myogenic competence cells into multipotent mesenchymal progenitor cells. It was also discovered to cause myoblasts to differentiate and become multipotent progenitor cells.[15] Another study showed that reversine can drive the development of lineage-bound mouse myoblasts into multipotent progenitor cells that can differentiate into osteoblasts or adipocytes under the right circumstances.[22] Thus, our finding add important knowledge of the benefit and capability of reversine to induce differentiation of human DFAT into cardiac derived cells, which can be usefull for further research and treatment for cardiovascular disease with stem cell approach.

This is inline with previous evidence that showed the benefit of reversine. Soltani et al. showed that treatment with very low concentrations of reversine promotes differentiation of DFAT cells.[23] Hiruma et al. also proved that dedifferentiated DFAT cells quantity were two to three times larger than cells that did not acquire reversine.

One of the possible effects of reversine in increasing the plasticity of DFAT cells is through the role of aurora B kinase. Previous research has found that polyploidy is caused by reversal-dependent interference with aurora B kinase activity, which is involved in a variety of processes such as mitotic chromosomal segregation, spindle checkpoint function, cytokinesis, and histone H3 phosphorylation.[24] Reversine treatment affects a network of genes that control cellular assembly and organization, DNA replication, recombination, and repair, cell-to-cell signaling, tissue morphology, as well as molecular transport and small molecular biochemistry, as demonstrated by Sabbattini et al. during postmitotic cell differentiation of MSCs.[25] Because of that, administration of reversine at its optimal dose might induce DFAT cells into a differentiated state and acquire characteristics such as mesenchymal stem cells possessing pluripotency through pathways dependent on chromatin remodeling, growth cycle arrest, and cell cycle regulation.

Our result showed that the effect of reversine in increase DFAT differentiation capability into cardiac derived cells was dose dependent effect, meaning the result was only statistically significant for spesific doses of reversine compared to control group without reversine added.

For VSMC differentiation, the significant reversine dose to increase α-SMA expression from DFAT cells in our study was on 10 nM and 20 nM. Some studies also support the possible mechanism of this result. Li et al. showed that in the process of vasculogenesis of VSMCs, the role of reversine initiated by Sonic Hedgehog (SHH) and WNT in the dedifferentiation process.[26] Another study showed that the fibroblast marker HSP47 progressively disappeared after reversine administration, supporting the possibility of differentiation into reversine-induced VSMCs.[27]

For CM differentiation, the significant reversine dose to increase cTnT expression from DFAT cells in our study was only on 10 nM. No previous study has shown that reversine is able to induce the differentiation of DFAT cells into CM cells. However, almost similar to our finding, Soltani et al. have proved that reversine increases the plasticity of mesenchymal stem cells in triggering the cardiomyocyte dedifferentiation process through cTnT expression.[23] There is a hypothesis that reversine's ability to induce differentiation is through the MEK/ER signaling pathway. This pathway has been shown to control many cellular processes, such as, metabolism, motility, cell proliferation, survival, apoptosis, in addition to stem cell dedifferentiation.[28]

For EC differentiation, the significant reversine dose to increase CD31 expression from DFAT cells in our study was on 10 nM and 20 nM. This result is the first to prove that reversine is capable of inducing of DFAT differentiation into EC cells. The mechanism is not yet clearly understood, however there is a possible role of angiogenic factor genes involved. Previous experimental study conducted culture of vascular endothelial cells and DFAT cells. The expression of HGF, FGF-2, and Ang1 genes in DFAT cells was significantly higher in co-cultured with vascular endothelial cells compared to controls.[13]

Our result also showed that the the three cardiac derived cells showed the same differentiation expression pattern after given reversine in various doses, that is increasing expression from control to dose 10 nM and to dose 20 nM reversine (small dose), and then decreasing at dose 40 nM reversine (large dose), with statistically significant in cTnT and CD31 expression. This shows that high doses of rerversine actually reduce the expression of cTnT, alpha SMA, and CD31 which indicates a different failure of the differentiation process compared to the control group. This is in line with some previous studies. Yu Guo et al. showed that reversine can provoke cell cycle arrest, ROS accumulation, and mitochondrial dysfunction in DFAT cells triggering the apoptotic process.[29] It was also stated that reversine can induce apoptosis of DFAT cells through the mitochondrial-mediated intrinsic pathway, which has shown similar effects in all types of mesenchymal cells.[30] This indicates that toxic dose of reversine induces cell cycle termination and promotes cell apoptosis of DFAT differentiation.

Conclusion

In conclusion, this study proves for the first time that there is a relationship between reversine and increased plasticity of human DFAT cells into cardiac-derived cells in the form of VSMCs characterized by the expression of α-SMA markers, CM characterized by the expression of cTnT markers, and EC characterized by the expression of the CD31 marker. The effect of reversine on differentiation of DFAT found in our study is biphasic, where it increases more expression of cardiac-derived cells in dose dependent effect on small dose (10 nM and/or 20 nM), and decrease expression in higher dose (40 nM). Further research is needed regarding the optimal dose of reversine and optimal passage for differentiation of DFAT cells into VSMCs, CM, and EC with a larger number of samples and replication, before being applied in in vivo assays. Cell physiology and interindividual and intraindividual variability in measurements need to be considered as confounders of the validity and reliability of the data.

Conflict of interest
The authors declare there is no conflict of interest.

Funding
None to declare.

Author contributions
MFR and BBD designed the study; MFR, YHO, and BBD accumulated the data; MFR, YHO, BBD and REI drafted the manuscript.

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