Cellular Therapy and Transplantation (CTT). Vol. 11, No. 2, 2022
doi: 10.18620/ctt-1866-8836-2022-11-2-72-83
Submitted: 27 May 2022, accepted: 24 June 2022

Co-culture of human cardiomyocyte and human amnion epithelial stem cells in amnion bilayer matrix for cardiomyogenesis

Muhammad A. Putra [1], Normalina Sandora [2], Tyas R. Kusuma [2], Nur A. Fitria [2], Tri W. Soetisna [3], Pribadi W. Busro [1], Ardiansyah [1], Chaidar Muttaqin [1], William Makdinata [1], Idrus Alwi [1]

[1] Department of Thoracic and Cardiovascular Surgery, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[2] Indonesia Medical Education and Research Institute, Human Reproduction Infertility and Family Planning Research Center, Jakarta, Indonesia
[3] Adult Cardiac Surgery Department, National Cardiovascular Center Harapan Kita, Jakarta, Indonesia

Dr. Normalina Sandora, Indonesia Medical Education and Research Institute, Human Reproduction Infertility and Family Planning Research Center, Jakarta, Indonesia
E-mail: normalinasandora@gmail.com

Citation: Putra MA, Sandora N, Kusuma TR, et al. Co-culture of human cardiomyocyte and human amnion epithelial stem cells in amnion bilayer matrix for cardiomyogenesis. Cell Ther Transplant 2022; 11(2): 72-83.

Summary

Our study developed a culture system to deliver cells for the regeneration of infarcted myocardial tissue. Human cardiomyocytes (hCardio) and human amnion epithelial stem cells (hAESC) were co-cultured in a biological scaffold forming a 3-D matrix, prepared as a heart patch candidate. The current investigation was aimed for assessment of hCardio-to-hAESC seeding ratio for the best conditions to provide cardiomyogenesis, i.e., 1:5 or the 1:6. This ratio corresponded to optimal cell number of 500,000 cells/cm^2, thus providing a 12-cm^2 heart patch. This cell ratio was also published in our previous study using human adipose stem cells and hCardio. The hCardio was isolated from the heart tissue taken during surgical correction performed in patients with right ventricular hypertrophy. The cell isolates expressed cTnT (10.7%), cKit/CD117 (16%), ICAM (94%), and PECAM+/VCAM- 33%. The hAESC was isolated from an elected donor at Caesarean section. It showed expression of TRA-1-60 (82.4%), SSEA-4 (28.2%), Oct-3/4 (2.9%), Nanog (11.4%), without expression of immune antigens, e.g., HLA-DR (0%), HLA-ABC (0.2%) as well as low-level expression of mesenchymal stem cell (MSC) markers, i.e., CD73 (20.2%), CD90 (0.4%), CD105 (59.2%). These cells did not exhibit Lin phenotype (CD3, CD14, CD16, CD19, CD20, CD56). At both seeding ratios, the co-culture matrix also released TNF-α at low levels (<1 pg/ml) throughout cultivation from day 1 to day 8. Both groups had shown a consistent growth of cells over time. Confocal images showed that the cell population has expanded and migrated to the deeper plane up to 140 μm after 5 days of incubation in both 1:5 and 1:6 groups. The cells seemed to connect and form projections, starting by day 5, being more obvious among the 1:5 group. They proliferated rapidly until no solitary cells were observed after day 8 of co-culture. Expression of the cardiomyogenesis genes, e.g., cTnT and ACTN2 in the 1:6 group were expressed, similarly to normal cardiomyocytes after eight days of cultivation. MHC genes in the 1:6 group were also expressed, although less than 1-fold to normal cardiomyocytes. This study indicated that hCardio and hAESC at 1:6 ratio seeded on amnion bilayer could support cardiomyogenesis derivation from the progenitor cells.

Keywords

Human amnion epithelial stem cells, human cardiomyocytes, cardiomyogenesis, amnion bilayer, 3-D scaffold, cell therapy.

Introduction

Coronary artery diseases are the second deadly disease worldwide causing heart failure as the terminal condition, claiming one life every 36 seconds in the USA [1, 2]. American Heart Association (AHA) reports a trend of coronary heart disease among youngsters as early as 20 years old, up to 15.5 million people, and experienced myocardial infarction every 46 seconds [3]. Similarly, according to the Indonesian Basic Health Research, Indonesia also shows an increasing tendency with 1.5% of the coronary heart disease cases at 15 years old or above, with related causes of death, 8.1% of general mortality in 2010. Total obstruction of the coronary artery causes death of cardiomyocytes, myocardial heart muscle infarction, and progressive failure when the vascularization is not immediately recovered. This condition has eventually led to heart failure, thus requiring heart replacement. In the Netherlands, only about 50% of patients received heart transplantation after 2.6 years of waiting, while 15% died [4].

Normally, the rate of cardiomyocyte replacement after birth is 1% *per* year after 20 years old, and 0.5% *per* year in the elderly. In total, 39% ventricular cardiomyocytes have been replaced after birth, while 36% of those cells have been replaced by 10 years old [5, 6]. Therefore, studies to regenerate the infarcted heart muscles are quite necessary, since the demand for heart transplantation is always insufficient. Nowadays, different attempts to improve cardiac regeneration capacity such as cellular transplantation, using different cell types such as stem cells and cardiac progenitor cells delivered using various methods. Stem cell therapy is believed to repair infarcted tissue, since the self-organ progenitor has been depleted [7, 8].

The available studies of cardiac engineering *in vitro* or *in vivo* point to low levels of newly formed cardiac tissue [9]. Animal studies and clinical practices presume this finding is due to low retention of the transplanted cells. Hou et al. (2005) have shown it by injections of human peripheral mononuclears (107 cells) labeled with ^{111}Indium-oxine in a porcine myocardial infarction model *via* intracardial, intracoronary, or intracoronary retrograde venous injections (IRV). Only 11.3% of the cells retained in the infarction area after six days after intracardiac injection, only 2.6±0.3% ($p<0.05$) were revealed locally following intracoronary delivery, and 3.2%±1%, when introduced by IRV [10].

Along with cellular retention, the issues in cellular therapy also include cell source, either autologous or allogeneic cells, which may be of low viability. Recently, tissue engineering has been proposed to improve cellular retention by using scaffolds to deliver the cells. Cell transplantation into the infarcted organ matrix seems to be irrational, since the matrix was subject to deterioration, it becomes thinner and stiff due to avascular structure and cell loss. The extracellular matrix composition has been altered, and the cardiac histoarchitecture has been disoriented. These alterations cannot provide a proper microenvironment for the newly transplanted cells. Hence, the cells may be delivered within a 3-D scaffold, as a provisional microenvironment to support stem cell colonization and growth [11]. The current tissue engineering technique is a co-culture system approach using a scaffold containing cells that can be applied to the damaged heart, known as "*scaffold-based cell therapy*" [12].

This study used human amniotic epithelial stem cells (hAESC) as a stem cell source for cell therapy, part of the translational study to regenerate the infarcted myocardium. Our group studied this cell type extensively *in vitro*, and the current study of co-culture with human cardiomyocytes had shown to induce the cardiomyogenesis process. Moreover, the origin of the hAESC presumes anti-inflammatory activity that concerns the issue of allogeneic tissue rejection, providing less immunogenic reaction. In addition, this is a waste tissue obtained at surgery, thus avoiding ethical issues, getting abundance of cells, and these samples are easy to obtain [13].

Materials and methods

Sampling of biomaterials

This study was designed to assess the optimal ratio of hAESC and hCardio for the cardiomyogenesis process when seeded on an amnion bilayer as the 3-D scaffold. All donors for human amnion epithelial stem cells (hAESC) and the human cardiomyocytes (hCardio) were obtained from the National Hospital Cipto Mangunkusumo, Jakarta, Indonesia. Placenta was obtained from the compliant donors negative for HIV, hepatitis B, and cytomegalovirus, by means of elective caesarean procedure which proceeded without complications. Placenta was transported to the laboratory within 30 minutes in the transport medium at 4°C. The transport medium consisted of DMEM (Dulbecco's Modified Eagle's Medium) 4.5 $g.L^{-1}$ (Sigma-Aldrich, USA), containing 10% (v/v) human platelet rich plasma (PRP), 1% (v/v) Inviclot® heparin sodium (Fahrenheit, Indonesia), and 3% (v/v) Gibco™ antibiotic-antimycotic (ThermoFisher, USA). The donor heart tissues were obtained from pediatric patients, with consent from their parents. The ethical board had approved all protocols of the Faculty of Medicine, Universitas Indonesia, and the ethical clearance number was KET.483/UN2.F1/ETIK/PPM.00.02/2019. Any specimens obtained in this study had been taken from the compliant donors, with informed consent obtained before the procedures.

Cardiac tissues were transported in the transport medium (see above), to the lab at 37°C to obtain the optimum cell number [14], while others were at 4°C unless otherwise stated, transported to the laboratory within 30 minutes. All specimens were initially verified for the microbial burden once they arrived at the lab, using a serial dilution of total plate count (TPC), taking the transport medium, inoculated onto Tryptic Soy Broth (Sigma-Aldrich, USA) and Difco™ Columbia Blood Agar (BD, USA) and incubated at 37°C. Also, inoculation onto Difco™ Sabouraud Dextrose Agar (BD, USA) was made to detect potential fungal contamination, with incubation at room temperature. All procedures to generate single-cells and culture were performed under aseptic conditions.

Isolation of the single-cell cardiomyocytes

The excess heart tissue was collected from the patients with hypertrophy of the right ventricle undergoing total

correction surgery to restore the volume of the heart chamber. According to the previous method developed, the protocol to isolate cardiomyocytes from heart tissue included enzymatic, thermal, and mechanical methods [15]. Briefly, once arrived at the lab, after the initial procedures stated above, the tissue was weighted, and only 500-1000 mg of tissue was taken. The sample was washed twice in Dulbecco's phosphate-buffered saline $Ca^{++}Mg^{++}$free (Sigma-Aldrich, USA), minced up to 2×2×2 mm^3, and loaded into a C-tube gentleMACS™ C-Tubes (Miltenyi Biotec, Germany), added with Collagenase type V (250 U.mL-1 Gibco™) (ThermoFisher, USA), and proteinase type XXIV (Sigma Aldrich, USA). The tube was then fitted to the sleeve of the gentle MACS™ Octo Dissociator with Heater (Miltenyi Biotec, Germany), incubated for 1 hour, 37°C. The digestion process was then neutralized using two volumes of AscleStem complete medium added with the supplement (Nacalai Tesque, Japan). The cells were harvested using a 70 µm cell strainer (Biologix, China) and pelleted at 600×g for 5 minutes at room temperature. The collected cells were stained with Trypan Blue dye to assess the cellular viability, counted in a Neubauer haemocytometer, and visualized using the Brightfield microscope AxioVert.A1 (Carl Zeiss, Germany). Further confirmation of cardiomyocyte by the cell shape was performed using Live/Dead assay (Invitrogen, USA) and visualized using LSM 900 (Carl Zeiss, Germany) [16].

Isolation of human amnion epithelial stem cells

The single-cell hAESC was isolated following the method described in several papers with modifications [17, 18]. After the bio-burden assay, the fetal membrane was spread on a sterile dish to separate the chorion. The amnion membrane was washed in 50 mL of Hank's Balanced Salt Solutions/HBSS (Gibco, USA), followed by 50 mL of Versene solution (Gibco, USA) twice, to remove blood residue. The amnion membrane was then spread with the fetal side upright, incubated with TripleSelect (Gibco, USA) 50 mL distributed evenly, for 1 hour, 37°C. Thereafter, the fetal side was scraped gently to remove the cell layer, followed by neutralization using DTI solution (1x volume). The cell suspension was separated from tissue debris using a 100 µm cell strainer (Biologix, China). The cells were then collected by centrifugation at 150 g for 10 minutes. The amnion membrane was flipped over to allow the maternal side to be upright, followed by incubation with 2 $mg.mL^{-1}$ Collagenase-1 (Gibco, USA) and Hyaluronidase 66$IU.mL^{-1}$ (Thermo Fisher, USA) in DMEM 4.5 $g.L^{-1}$ (Sigma-Aldrich) for 1 hour, 37°C. The cell suspension was then filtered using a 100 µm cell strainer (Biologix, China); the cells were then harvested by centrifugation of the filtrate at 150 g, 10 minutes. The digestion was stopped using AscleStem™ Cardiomyocyte Differentiation Medium (Nacalai Tesque, Japan) complete medium, and the isolated cells were then collected, as mentioned before.

Characterization of the isolated hAESC and hCardio

All primarily isolated single-cell preparations were characterized using flow cytometry, antibodies used to identify the specific cells according to the specific epitopes described in the literature review. The epitopes were identified using BD FACSAria™ III, and the data were analyzed using BD FACS Diva 8.0.2 (BD Bioscience, USA). The cells of interest were separated from the background noise according to the data from unstained reading; the cut-off lower limit of the interested populations was set to the highest limit of the corresponding isotype specific to each antibody.

Both cell types, i.e., hAESC and hCardio, were firstly labelled for mesenchymal stem cell identity [19] against CD73 (Biolegend, USA), CD90 (Biolegend, USA), and CD105 (Biolegend, USA), whereas the anti-human Lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56) or Lin- (Biolegend) was used as the negative index. Further immunomodulatory factors in hAESC were confirmed to their HLA-DR antibody (L243) PE (SantaCruz, USA) paired to the murine monoclonal IgG2a PE, and the HLA-A/B/C antibody (D-2) FITC (SantaCruz, USA) paired to the mouse monoclonal IgG1 FITC (SantaCruz, USA). The h-Cardio were identified by labeling for the cardiac Troponin (cTnT) FITC (SantaCruz, USA) [20] paired to the murine monoclonal IgG2a kappa (SantaCruz, USA), murine anti-human CD31/ PECAM-1 FITC (BioLegend, USA) [21], and murine IgG-FITC isotype (BioLegend, USA), anti-human CD106/ VCAM1 PE (Biolegend, USA) paired to mouse IgG1 kappa isotype (BioLegend, USA), CD117/ cKit PE (Miltenyi Biotec, Germany) with mouse IgG1 kappa PE isotype (BioLegend, USA), CD 54/ ICAM PE (SantaCruz, USA) with mouse monoclonal IgG1 kappa isotype (BioLegend, USA). The hAESC were determined to the Nanog (A-11) PE (SantaCruz, USA) paired to mouse monoclonal IgG1 kappa PE (SantaCruz, USA) [17], Oct-3/4 (C-10) FITC (SantaCruz, USA) paired to mouse monoclonal IgG2b FITC, TRA-1-60 PE (SantaCruz, USA) paired to the murine monoclonal IgM PE, and SSEA-4 (813-70) FITC (SantaCruz, USA) paired to mouse monoclonal IgG3 FITC [18].

The cells prepared for flow cytometric analysis were set at 10^5 cells *per* reading. All antibodies were labelled using extracellular markers, except the c-TnT labelling, since these epitopes are located at the wall of the nucleus membrane. The intracellular labelling followed the protocol published elsewhere. Briefly, after 2x washes with Ca^{++}, Mg^{++} free PBS (10,000 rpm 10 min, the cells were washed in detergent to disintegrate the cell membrane using BD Perm/Wash™ (1 ml) at 25°C for 15 minutes, followed by fixation using BD Cytofix™ Fixation Buffer (4 mL), at 25°C, 15 min, washed in PermWash (2x). Then the pellet was labelled against cTnT, incubated at 4°C, for one hour. The extracellular labelling was performed with PBS Ca^{++} Mg^{++} free consecutively after each treatment, labelled with all antibodies and isotypes stated above with 1 µl *per* 10^6 cells, at 4°C for 1 hour. The cell population was counted using FACS BD Flow cytometer, designed to stop after 30,000 cells counting.

Co-culture of hAESC and h-Cardio on an amnion bilayer scaffold

Both cell types were seeded onto the amnion bilayer, with a seeding density of 5×10^5 $cell.cm^{-1}$. The ratio of hAESC and h-Cardio seeded on the graft was prepared at a 5:1 ratio [22], as the maximum number of cells isolated from each harvest was about 10^6 cells; therefore, the optimum cell ratio was compared to the 1:6. The mixture cells were suspended in the complete AscleStem™ Cardiomyocyte Differentiation Medi-

um (Nacalai Tesque, Japan). Briefly, the amnion bilayer scaffold was round in shape, sized 4 cm in diameter, blotted dry and placed inside-out, seeded with cell-mixture suspension at 5×10^5 cell.cm^{-1} diluted in the complete AscleStem™ Cardiomyocyte Differentiation Medium (Nacalai Tesque, Japan); with addition of Supplements A (Nacalai Tesque, Japan and B (Nacalai Tesque, Japan). The seeded graft was incubated at 37°C, 5% CO_2, for nine days, and the culture medium was refreshed every three days.

The seeded co-cultures of hAESC and h-Cardio were terminated after Day 2, Day 5, and Day 8 incubation for proliferation and cardiomyogenesis verifications.

Cellular proliferation

The proliferation of the seeded cells was counted by the ATP amounts generated by the cells, quantified using ATPLite assay (PerkinElmer, USA), and qualitatively using Live/Dead staining (Invitrogen, USA), and visualised using LSM 900 (Carl Zeiss, Germany). The ATP contents have been measured by counting the luminescence intensity *per* second using the plate-reader Varioskan™ LUX multimode microplate reader (Thermo, USA). Briefly, each graft was washed in DPBS 3x, then minced and digested in lysis buffer (Perkin Elmer, USA) for 10 min, with agitation at 25°C, the tissue was separated by centrifugation. 100µL of the suspension were aliquoted into each well of the Opti-Plate well (Thermo, USA), with replications, supplied with 100µL of Luminescence buffer and shaked at 300 rpm for 10 minutes in the dark.

Qualitative viability analysis was determined using Live/Dead staining (Invitrogen) [23]. Briefly, the specimens were washed three times in DPBS (Sigma, USA), and incubated in Calcein 4µM that will stain cytoplasm of viable cells, and Ethidium homodimer (2 µM) staining nuclei of the dead cells, for 30 minutes, 37°C, in dark, followed by DPBS (Sigma, USA) washes (three times). The specimens were placed on a microscope slide, added with DABCO™ mounting medium (Sigma, USA), and visualized under the confocal microscopy, LSM900 confocal microscope (Zeiss, Germany).

Real-time RT-qPCR

The cardiomyogenesis gene expression from the seeded co-culture of the h-Cardio and hAESC in a 3D amnion bilayer graft were analyzed using RNA extracted with TRIzol™ Reagent (ThermoFisher, USA). RNA samples (1 µg) were converted to the complementary matrix DNA using SensiFAST™ cDNA Synthesis Kit (Bioline, USA), then amplified using Techne Prime Pro 48 Real-time qPCR System (Techne, UK), SensiFAST™ SYBR Lo-ROX Kit (Bioline, USA). The α-actinin expression identified the specific feature of cardiomyogenesis process (at the Z-disc, this factor regulates transcription of the actin-binding protein, and contraction of myofibrillar actin filaments) [24]. The primers used were as follows: for actinin (ACTN2): F5'AGCCGAATTTGCCCGCATTA3', R5'TGAAGGATTGGAAGGTGACGG3'; for cTnT (cardiac regulatory proteins to coordinate the interaction between actin and myosin through the calcium channel) [25], the human cardiac troponin/ cTnT: F5'CAAGGAGCTGTGGCAGACGAT3', R5'GCTCCCCATTTCCAAACAGGA3', and for the myosin heavy chain/MHC (reflecting the contractility of cardiac muscle) [26] MHC: F5'ACCTGAAGGAGAACATCGCC3', R5'AAGCCCTTCGTGCCAATGTC3'. The reference gene was GAPDH detected with the following primers: F5'AACATCATCCCTGCCTCTACT3', R5'CTCCGACGCCTGCTTCAC3'.

Data analysis of the RT-PCR was performed using the Livak method. Briefly, all ΔCT values were normalized to the Ct value of the reference gene, followed by normalization of the cardiomyogenesis genes (cTnT, MHC, α-actinin) to the cardiomyocytes of normal heart serving as controls, to obtain the ΔΔCT values.

TNFα measurement using ELISA

The measurement of TNFα cytokine released to the culture medium after incubation of the co-culture was made from Day 1, Day 5, Day 7, and Day 8. H9C2 cells (rat neonatal heart cells) seeded in each 96-well plate, 25,000 cells *per* well, after 24 hours incubation with DMEM containing 10% FBS (Gibco, LifeTechnologies), were replaced with the tested culture medium and allowed to incubate for 48 hours. The measurements were performed using ELISA kit (1-StepTM Ultra TMB-ELISA, Thermo Scientific).

Results and discussion

This study is a part of the translation study to regenerate an infarcted heart wall using scaffold-based cell therapy. Cell therapy using a co-culture of hAESC and h-Cardio delivered in an amnion bilayer as a vehicle and temporary media before the transplanted cells will adapt for the new microenvironment. Our aim was to derive the hAESC from becoming the self-cardiomyocytes. As cell therapy is considered a tool for regeneration of infarcted heart wall, this study aims for helping the physicians to decide which cell source is suitable for the patient, either autologous or allogeneic cells.

The 3-D matrix vehicle used in this study was an amnion bilayer (Fig. 1A), prepared using a decellularized amnion membrane after extensive washes in 0.05% SDS (w/v) and Triton X-100, 0.1% (v/v), then overlayed with fibrin. The acellularity was confirmed using H&E staining (Fig. 1B), since no nuclei were identified, and further DNA residue was validated using DAPI staining, with no blue fluorescence highlighting the double strain DNA (Fig. 1C). The graft was biocompatible as revealed by contact toxicity assay using human bone marrow-MSC, with cell growth into the matrix (Fig. 1D). Fig. 1E shows the waste tissue of the heart weighing about 500-1000 mg. The tissue was then processed to become a single cell, shown in Fig. 1F, as the rod-shaped cells reported in many papers [27]. Fig. 1G shows the single cell of hAESC with a cobblestone appearance.

The heart cells were then characterized using flow cytometry to identify the cardiomyocyte phenotypes, e.g., cTnT (10.7%), progenitor cell marker cKit (16%), the adhesion molecule ICAM (94%), the endothelial marker PECAM+/VCAM- (33%), as seen in Fig. 1H. The hAESC were shown to express pluripotent phenotypes: TRA-1-60 (82.4%), SSEA-4 (28.2%), Oct-3/4 (2.9%), Nanog (11.4%), with no detectable expression of immune antigens, such as HLA-DR (0%), HLA-ABC (0.2%). Expression of mesenchymal markers was

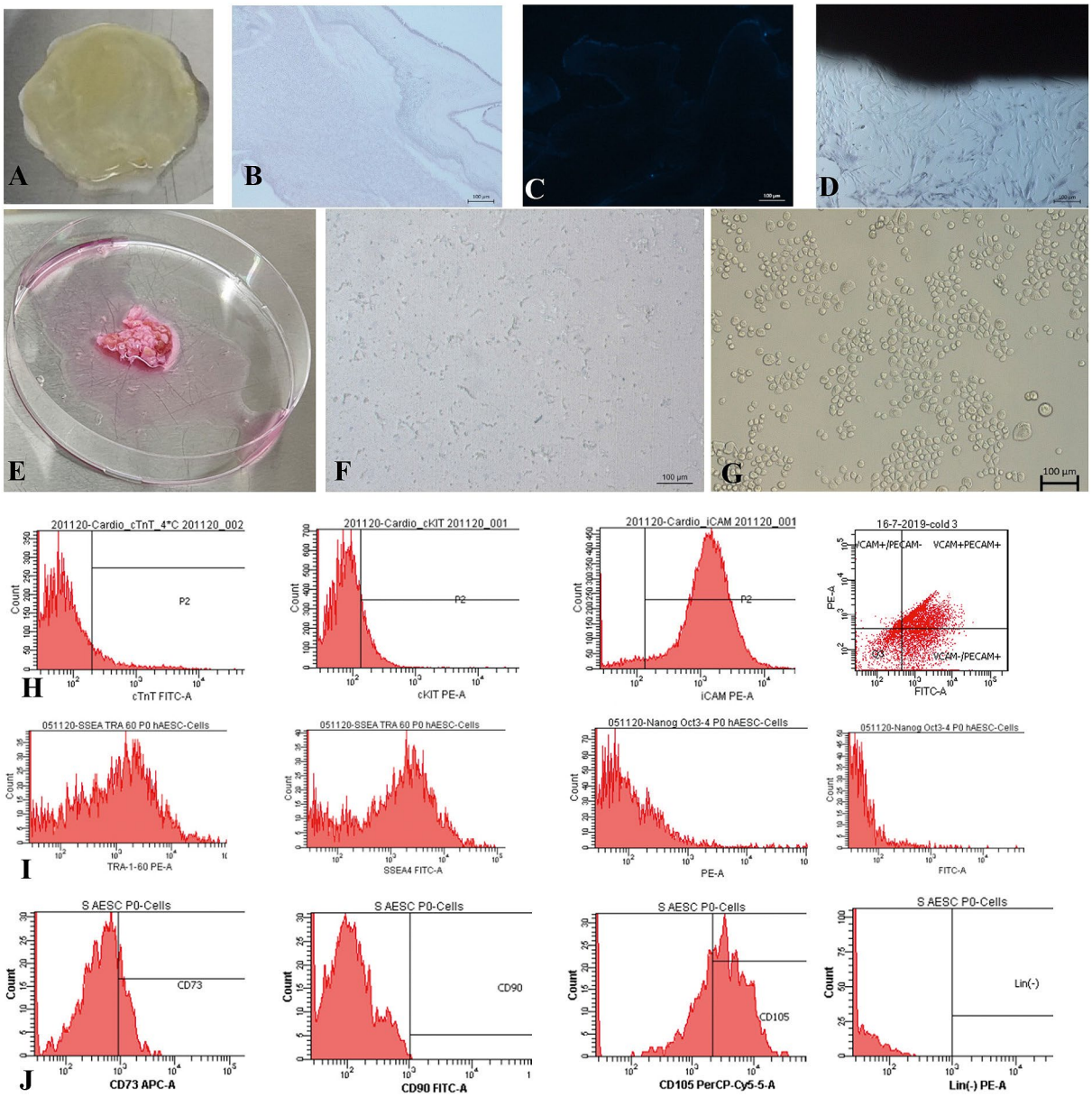

Figure 1. The scaffold for 3-D culture (amnion bilayer) used in this study. (A) Macroscopic view of the scaffold, thickness of 500 µm. (B) Section of the amnion bilayer stained with H&E and (C) DAPI, showed no cell remnants, or traces of DNA, respectively. (E) The waste tissue taken from the excess heart wall. The single cells of the (F) hAESC and (G) hCardio following isolation from the human amnion membrane and cardiac samples, respectively. The cells were captured at 100x magnification.

low, i.e., CD73 (20.2%), CD90 (0.4%), CD105 (59.2%), while the Lin- (CD3, CD14, CD16, CD19, CD20, CD56) were undetectable (Fig. 1I, 1J). Many studies have already reported these expression patterns, among other embryonic stem cells markers SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, Oct-4, and Nanog [18]. Therefore, the hAESC might be a suitable candidate for transplantation taken from allogeneic sources, because they had no expressed antigen that would induce immune-mediated rejection [28, 29]. Further investigations will show whether this cell candidate and the proposed culture system are suitable for deriving cardiomyogenesis.

The production of the pro-inflammation cytokine released from the transplant or donor tissue might hinder the transplant from integrating at the transplantation site. The proinflammatory cytokines TNF-α and IL-12 are released by cytotoxic M1 macrophages (CD68+ and CD80+) related to production of highly reactive oxygen intermediates and nitric oxide. These classic factors of inflammation method kill the pathogens; however, they sometimes cause the reverse effect to the host through massive fibrosis or scarring [30]. Some reports showed that elevation of TNF-α after transplantation correlates with fibrosis/scarring process in pulmonary fibrosis [31], and scarring trachoma [32]. Other workers also reported that primary cells from the hAESC at passage 0 until passage 5 suppressed the T cell proliferation by exerting immunosuppressive effects [33].

The current study showed that the co-culture of hAESC and h-Cardio at both ratios (1:5 and 1:6) caused only weak release of TNF-α into the culture medium (<1 pg/mL) after 1-5-7-8 days of cultivation. There were no differences between the both groups at any time point (Fig. 2B). The data on TNF-α serum levels among patients after liver

transplantation showed that TNF-α was significantly higher among the patients with organ rejections (941±83 pg.mL^{-1}) compared to the patients in a stable clinical condition (240±6 pg.mL^{-1}; p=0.0001) [34]. Hence, the measurement of the culture medium of the co-culture hAESC and h-Cardio in the amnion bilayer graft suggests that the co-culture might not induce inflammation under the *in vivo* conditions.

The culture system in a custom-made 6-well plate to incubate the co-cultures in a 3-D construct is shown in Figure 2A. The Brightfield images of the co-culture constructs (Figure 2 C-G for the 1:5 ratio, and the Figure 2H-L for the 1:6 group; Day 1-3-5-7-8, respectively).

The confocal microscopy images (Figure 3A-C, 1:5 ratio, and Figure 3D-F, 1:6 ratio; Day 2-5-8, respectively) also demonstrate that the cell population had consistently expanded over time. The confocal images of the co-culture cells stained with Calcein AM were visualized using LSM700 Observer 2.1 (Zeiss, Germany), Z-stack every 10 µm. The initial culture on Day 2 showed that the cells were still solitary, rounded, and aggregated. The cells looked well distributed across the plane, although still sparse. These might indicate that the seeding method provided good distribution. On day 5, the cell population started to proliferate with increase of cellular population, and, apparently, some cell-to-cell connections were seen. The cells also migrated by ingrowth to the deeper layers, until 120 µm, compared to only 70 µm for the 1:5 group (Figure 3A, B, C), the 1:6 group (Figure 3D, E, F) also had migrated to the deeper plane. After 8 days of incubation, all experimental groups showed projections between cells (T-tunnel/ TNT) behaviour, and the population became very dense. It was unable to discern single cells. The 1:5 and the 1:6 images appeared as the cell colony (Fig. 3C and F, respectively). This cytoplasm projections are believed to facilitate cell-to-cell communications. Dupont et al. (2018) reported that the protrusion of the tunnelling nanotubes (TNT) is formed by the projection of the thin cell membrane to reach the distant cells, or through exosomes or vesicles [35]. Other reports showed that direct co-culture of mixed cells creates cell communications between different cell types through a gap junction and other means such as cytokine or hormone effects through specific receptors [36].

In general, cellular survival depends on their capability to adhere and consolidate to the surrounding extracellular matrix, which is facilitated by the cellular adhesion peptides (CAP), such as the RDG binding molecules. Fibronectin is one of the RGD that decides the re-endothelialization of the scaffold through integrin α5β1 and αvβ3 dimers binding [37]. Furthermore, the optimizing integrin, heparin, and leucocyte surface receptors may improve cellular integration into the matrix *in vitro* [38].

The current study investigated relative gene expression in cardiomyogenesis by qPCR using the following markers: cardiac troponin (cTnT), myosin heavy chain (MHC), and α-actinin (ACTN2) expression. The co-culture on day 2 and day 5 did not show any cardiomyogenesis gene expression in both cultures. Nevertheless, since day 8, the 1:6 group had expressed the specific gene for cardiomyocytes; the cTnT (one-fold), the MHC, and the ACTN2 (one-fold) (Fig. 4).

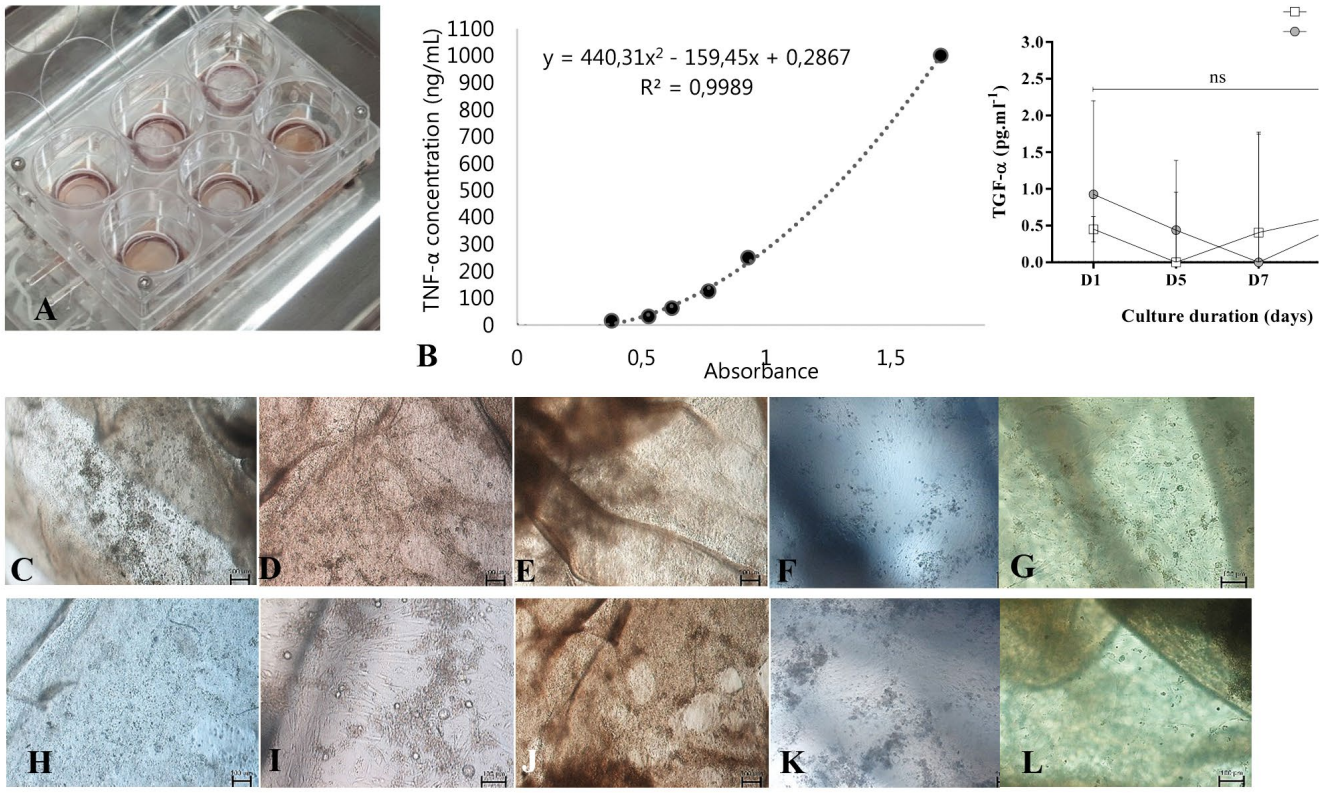

Figure 2. The images of 3D culture system. (A) the 6-well plate modified to incubate the seeded hAESC and hCardio on the amnion bilayer. (B) Measurement of TNFα and its standard; 3-D culture of hCardio and hAESC; (C,H) Day 1, (D,I) Day 3, (E,J) Day 5, (F,K) Day 7, (G,L) Day 8. The top row (C-G) shows the cultures at 5:1 ratio, and the bottom row (B-L) shows 6:1 ratio. D and I are made at 50x magnification, others show 100x magnification.

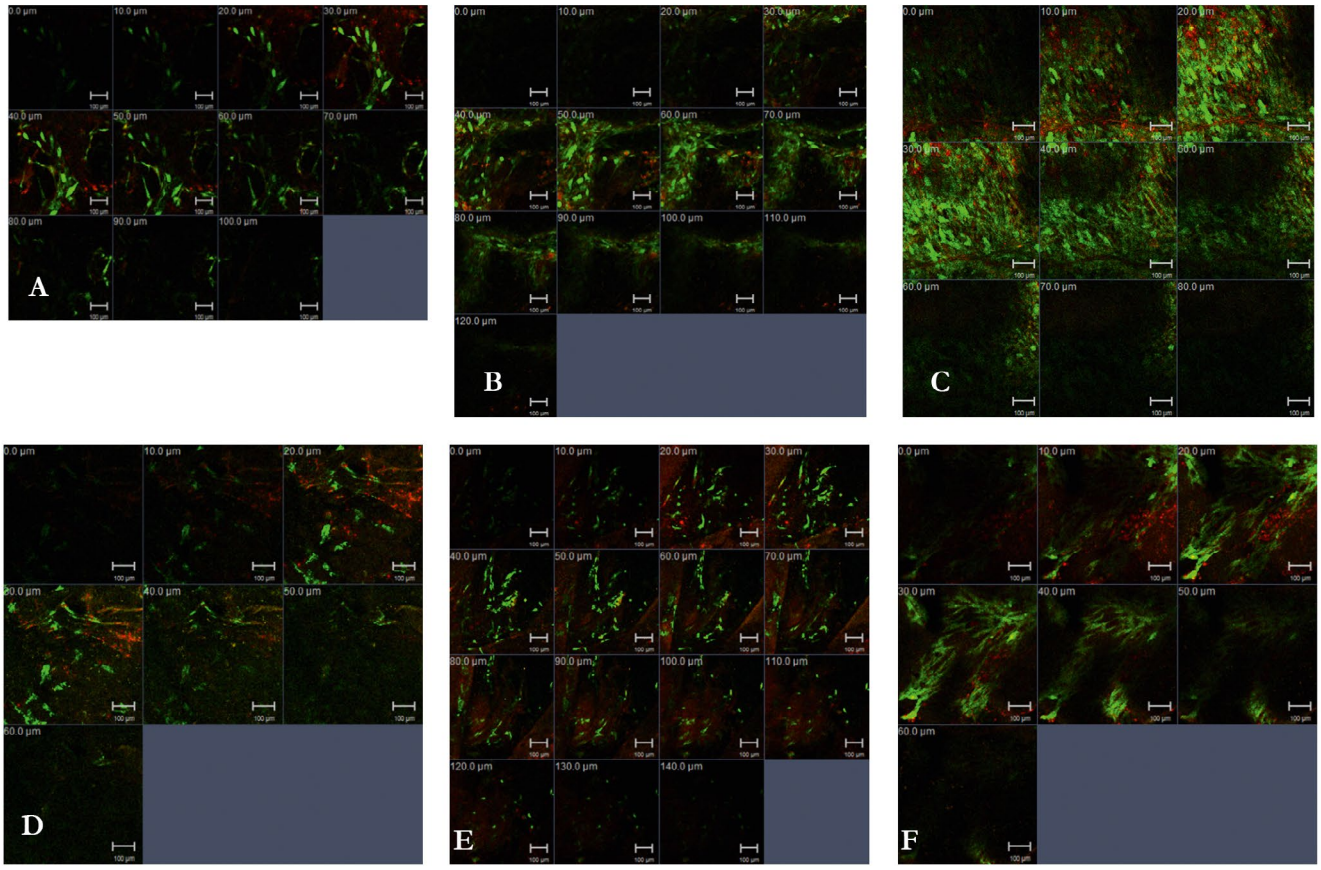

Figure 3. The 3-D images of hAESC co-culture with hCardio in amnion bilayer graft on Day 2 (A,D), Day 5 (B, E), and Day 8 (C, F). The A-C present the 1:5 seeding ratio, and the D-F were the 1:6 ratios

Viability assay was performed with Live/Dead staining, the images were taken using a confocal microscope 700 LSM Observer 2.1 (Zeiss, Germany) using Z-stack, at the interval of 10 µm. Green colour indicates viable cells, stained with Calcein AM (excited at 480 nm). Red colour indicates dead cells, stained using Ethidium homodimer (excited at 555 nm. emission at 560 nm), with laser diode gained at 635 nm.

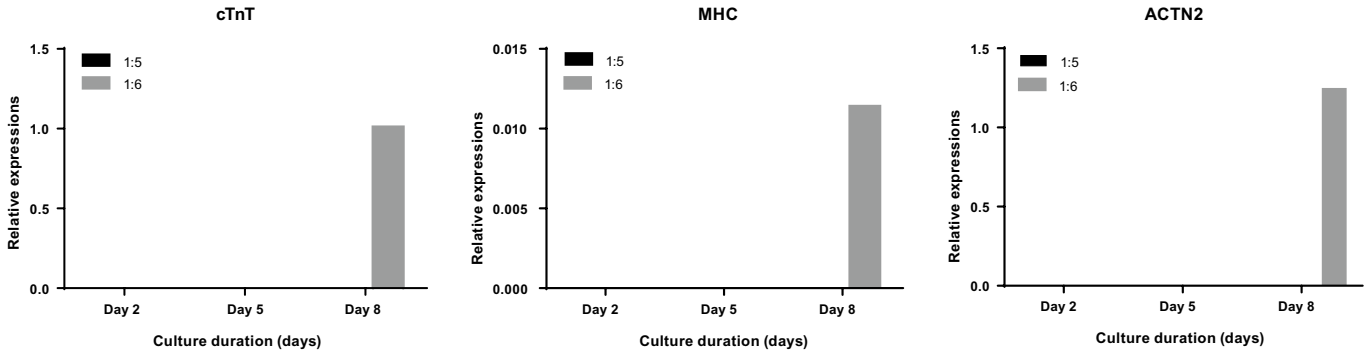

Figure 4. Gene expression in the 3-D co-cultures of the hAESC and hCardio after 2, 5 and 8-day incubation

All samples showed no differences after analysis by the two-way ANOVA test, and compared using Sidak's test. Although the "1:6" group after 8-day incubation showed slight increase compared to others, this difference was insignificant. The difference between the groups at different seeding ratios was insignificant for cTnT expression (p=0.42), MHC (p=0.42), and ACTN2 (p=0.44), neither the culture duration (p>0.05).

Perhaps, the co-culture of h-Cardio and hAESC at 1:6 ratio on a 3-D amnion bilayer scaffold might be the optimal culture condition to achieve cardiomyogenesis. Meanwhile, our previous study of a 2-D culture of h-Cardio and hAESC revealed absence of cTnT, MHC, and ACTN2 expression (unpublished results).

Adult cardiomyocytes have limited ability to regenerate after birth [5,6]. Therefore, adding a suitable cell type to improve severe heart diseases, such as progressive heart failure, is essential. In the present study, the waste heart tissue from the patients aged 0-2 years old subjected to the total correction surgery due to the tetralogy of Fallot was used for the

cardiomyocyte isolation. The isolated cardiomyocytes supplied with hAESC to augment the transplanted cells were delivered to the 3-D scaffold. The scaffold acts as a temporary shelter that provides an ideal microenvironment for the transplanted cells until the cells adapt to the surrounding host environment. The microenvironment in an injured myocardium seems not provide effective support to the cell survival due to hypoxic and low-glucose conditions. The *in vitro* modeling of an infarct condition has shown to restrain cellular viability and prevent the stem cells from differentiation to cardiomyocytes [39]. In conclusion, optimal results from cell transplantation are not only the matter of cells, but a correct delivery approach is also crucial. In this respect, tissue engineering might resolve this problem.

Many reports have indicated that hAESC expressed cardiac-specific genes encoding atrial and ventricular myosin light chain 2 (MLC-2A and MLC-2V), and the transcription factors GATA-4 and Nkx-2.5 *in vitro*. Expression of MLC-2A and MLC-2V, as well as GATA-4 and Nkx 2.5 was shown by RT-PCR in hAESC after 14 days of cultivation in the medium supplemented with 1 mM of ascorbic acid. The hAESC differentiated to cardiomyocytes exhibited expression of α-actinin, as proved by immunocytochemistry [40]. Other studies comparing ability of hAESC, human umbilical cord cells, and human adipose MSC for regeneration of myocardial infarction following intramural injection in athymic nude rats have found that hAESC was superior to other cells types, i.e., after four weeks, 3% of the injected hAESC exhibited a myocardial marker. The area of infarction after hAESC injection was characterized by decreased infarct size and showed an improved cardiac function [41].

Nonetheless, other cells, such as bone marrow MSC, are also believed to have capability for cardiomyogenesis. Bone marrow MSCs were taken from the femur and tibia of rats and co-cultured indirectly with cardiomyocytes taken from the ventricular wall at the 1:10 ratio, separated with a semipermeable membrane, and then cultivated for 1-3 weeks. It was shown that the resulting cells expressed SERCA2 and RyR genes, and, when implanted in rats, cardiac troponin T, cardiac troponin I, α-actinin, and desmin were expressed in the sections [42].

Direct contact between the cells is required to stimulate the differentiation of hAESC into cardiomyocytes. It is believed that bioelectric signals between cells dictate cell signalling that influences the resting potential of cell membranes which controls regulation of growth, migration, and differentiation signals. These findings were reported in a previous study by Wang et al. concerning the factors that encourage cell differentiation into cardiomyocytes through direct contact: the elasticity of cell, increase of ligands-receptors, molecule-integrin adhesion, and signal transcription [43].

The author realized that no identical study uses similar cell types and culture systems to compare. A study from our group using human adipose MSC taken from a younger donor (30 years old) compared to the elderly donor (60 years old) when co-cultured with h-Cardio on a 3-D scaffold (at 1:5 and 1:6 ratio, respectively), showed that initial culture (Day 2) only the 60 years old group had slight expression of MHC. However, after Day 5, the 30-year old group was superior in expressing cTnT, MHC, ACTN2, and, even, PPARγ, at significantly higher level, but it was diminished after Day 9 in all groups [44]. That study reported that the 1:5 ratio for adipose MSC is superior, as it revealed higher numbers of viable cells. Adipose MSC is considered an excellent autologous cell source, being less invasive for sampling. Nonetheless, according to Sandora et al. (2021) it might not be an ideal cell source when the elderly patient occurs with myocardial infarction. It was shown that, even though the elderly group was taken from the brown adipose tissue and had higher MSC expression than in younger persons (30 years old, white adipose tissue), the cardiomyogenesis process was superior when using the cells from younger donors. However, this study cannot maintain a cardiomyogenesis observed in this system after Day 9, as all the interested expressions disappeared [44].

Nonetheless, another co-culture study also reported similar results to our study; however, using an indirect co-culture system of adipose MSC and cardiomyocytes studied in rats, separated by a semipermeable membrane at the ratio of 1:5 for 2 weeks, indicated that the MSC transformed into elliptical-shape cells and started to contact with other cells to form a streaky pattern. The cells were found to express α-actinin and cardiac troponin (cTnT) just after 5 days of incubation and reached their peak by the day 14 (29.63% and 27.38%, respectively). The medium was also found to contain TGF-β, GATA-4, Nkx-2.5, and MEF-2C which pointed to the cardiomyogenesis process [22, 45]. The human heart expresses β1- and β2-adrenergic receptors that are crucial in developing heart failure [46]. The β1 subtype stimulates cardiac muscles but relaxes blood vessels, while the β2 subtype plays an opposite role; in relaxing the smooth muscle and regulating the contractility of cardiac muscles [47]. Ageing events affect sensitivity of β-adrenergic receptors in elderly persons (59-71 years old) compared to young subjects (1-13 years old) [48].

There are two methods to deliver cell or cell vehicles in cardiac engineering, e.g., using a heart patch or hydrogel carrier. There are advantages and disadvantages between these two methods. The patches are more invasive and require open heart surgery; however, they can be transplanted in any area that cannot be reached transthoracic, firmly stitched to the epicardial. The hydrogel can be delivered *via* a catheter, being less invasive. However, the accessible area to deliver the cells is more limited than with the patches [49]. In this study, the amnion bilayer was designed as a heart patch. The use of a 3-D scaffold derived from acellular amnion membrane biomaterials (amnion bilayer) is a tissue by human origin, being acellular and still maintaining high elasticity. The matrix also contains fibronectin (not published), which increases stem cell adhesion and growth [50]. These properties might provide a suitable microenvironment for the transplanted cells. Along with the biological scaffold, a bioactive matrix might also be a promising candidate to improve cellular retention when the cells are transplanted in order to regenerate the infarcted heart wall [52].

Human amniotic epithelial cells (hAESCs) possess embryonic stem cell-like proliferation and differentiation capabilities and adult stem cell-like immunomodulatory properties [40]. As described in an extensive review, hAESC is considered a

suitable candidate that can differentiate into cardiomyocytes [52]. It also do not express HLA-DR and HLA-A, B, C, thus preventing induction of immune reaction when transplanted [28, 29]. Compared with other stem cells, hAESC has unique advantages, including easy isolation, abundant cell quantities, avoiding ethical debates, as well as lower immunogenicity and absence of tumorigenic properties [53]. The cultivated and isolated hAESC populations are also reported to express normal karyotype, and are nontumorigenic upon transplantation [40].

Myocardial infarction (MI) leads to heart failure (HF) due to cardiac remodelling, especially wasting of the left ventricle (LV). The remodelled heart wall wastes the structure, molecular function, heart cells, and heart size, shape, and undergoes functional deterioration. Patients with extensive remodelling areas will immediately progress to terminal heart failure. Recent anti-remodelling therapy only slows the heart failure progression, nonetheless, the morbidity and mortality are still high [54].

Conclusion

This *in vitro* study showed that options for cell therapy (h-Cardio co-culture with hAESC at 1:6 ratio) using a biological scaffold (amnion bilayer) exhibited activation of the cardiomyogenesis-associated genes. The co-cultured cells were able to attach, migrate, proliferate and form intercellular connections. In future studies, the h-Cardio will be taken from the patient's autologous cells, whilst the hAESC will be from an allogeneic source.

Acknowledgement

The authors would like to thank Dr. Cipto Mangunkusumo National Central Public Hospital for facilitating the authors in subject recruitment and Faculty of Medicine.

This study was supported by grant no. NKB-0541/UN2.R3.1/HKP.05.00/2019 from the University of Indonesia.

Conflict of interest

The authors declare that there is no conflicts of interest.

Ethical approval

All methods in this study have been reviewed and approved by the Ethical Committee of Universitas Indonesia with Ethical Approval no. KET.483/UN2.F1/ETIK/PPM.00.02/2019.

All methods in this study were conducted in accordance with the Ethical Committee of Universitas Indonesia (Ethical Approval no. KET.483/UN2.F1/ETIK/PPM.00.02/2019) approved protocols.

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

References

1. Ralapanawa U, Sivakanesan R. Epidemiology and the Magnitude of Coronary Artery Disease and Acute Coronary Syndrome: A Narrative Review. J Epidemiol Glob Health. 2021;11(2):169-177. doi: 10.2991/jegh.k.201217.001

2. Control CfD, Prevention. Underlying cause of death, 1999-2018. CDC WONDER Online Database Atlanta, GA: Centers for Disease Control and Prevention. 2018.

3. Sanchis-Gomar F, Perez-Quilis C, Leischik R, Lucia A. Epidemiology of coronary heart disease and acute coronary syndrome. Ann Transl Med. 2016;4(13):256. Epub 2016/08/09. doi: 10.21037/atm.2016.06.33

4. Roest S, Kaffka genaamd Dengler SE, van Suylen V, van der Kaaij NP, Damman K, van Laake LW, et al. Waiting list mortality and the potential of donation after circulatory death heart transplantations in the Netherlands. Netherlands Heart Journal. 2021;29(2):88-97. doi: 10.1007/s12471-020-01505-y

5. Michler RE. Stem cell therapy for heart failure. Methodist DeBakey cardiovascular journal. 2013;9(4):187-194. doi: 10.14797/mdcj-9-4-187

6. Lázár E, Sadek HA, Bergmann O. Cardiomyocyte renewal in the human heart: insights from the fallout. Eur Heart J. 2017;38(30):2333-2342. doi: 10.1093/eurheartj/ehx343

7. Smit NW, Cocera Ortega L, Vegh AMD, Meijborg VMF, Smits AM, Klerk M, et al. Human cardiomyocyte progenitor cells in co-culture with rat cardiomyocytes form a pro-arrhythmic substrate: evidence for two different arrhythmogenic mechanisms. Front Physiol. 2017;8:797. doi: 10.3389/fphys.2017.00797

8. Ghiroldi A, Piccoli M, Cirillo F, Monasky MM, Ciconte G, Pappone C, et al. Cell-based therapies for cardiac regeneration: a comprehensive review of past and ongoing strategies. Int J Mol Sci. 2018;19(10). doi: 10.3390/ijms19103194

9. Joggerst SJ, Hatzopoulos AK. Stem cell therapy for cardiac repair: benefits and barriers. Expert Rev Mol Med. 2009;11:e20. doi: 10.1017/s1462399409001124

10. Hou D, Youssef EA, Brinton TJ, Zhang P, Rogers P, Price ET, et al. Radiolabeled cell distribution after intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery: implications for current clinical trials. Circulation. 2005;112(9 Suppl):I150-1156. doi: 10.1161/circulationaha.104.526749

11. Taylor DA, Chandler AM, Gobin AS, Sampaio LC. Maximizing cardiac repair: should we focus on the cells or on the matrix? Circ Res. 2017;120(1):30-32. doi: 10.1161/CIRCRESAHA.116.309959

12. Rodrigues ICP, Kaasi A, Maciel Filho R, Jardini AL, Gabriel LP. Cardiac tissue engineering: current state-of-the-art materials, cells and tissue formation. Einstein (Sao Paulo). 2018;16(3):eRB4538. doi: 10.1590/S1679-45082018RB4538

13. Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, et al. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. Stem Cells. 2008;26(2):300-311. Epub 2007/11/03. doi: 10.1634/stemcells.2007-0594

14. Putra MA, Sandora N, Suwarti, Nurhayati RW, Nauli R, Kusuma TR, et al. Transport viable heart tissue at physiological temperature yielded higher human cardiomyocytes compared to the conventional temperature. Cell Tissue Bank. 2022. doi: 10.1007/s10561-021-09978-w

15. Sandora N, Putra MA, Nurhayati RW, Suwarti, Nauli R, Kusuma TR, et al. Characterisation of the single-cell human cardiomyocytes taken from the excess heart tissue of the right ventricular outlet in congenital heart disease. Cell Tissue Bank. 2021. doi: 10.1007/s10561-021-09970-4

16. Axio Imager 2 Microscopy [Internet]. Carl Zeiss Microscopy LLC. 2013.

17. Koike C, Zhou K, Takeda Y, Fathy M, Okabe M, Yoshida T, et al. Characterization of amniotic stem cells. Cellular Reprogramming. 2014;16(4):298-305. doi: 10.1089/cell.2013.0090

18. Miki T, Mitamura K, Ross MA, Stolz DB, Strom SC. Identification of stem cell marker-positive cells by immunofluorescence in term human amnion. J Reprod Immunol. 2007;75(2):91-96. doi: 10.1016/j.jri.2007.03.017

19. Germscheid NM, Thornton GM, Hart DA, Hildebrand KA. Wound healing differences between Yorkshire and red Duroc porcine medial collateral ligaments identified by biomechanical assessment of scars. Clin Biomechanics. 2012;27(1):91-98. doi: 10.1016/j.clinbiomech.2011.07.001

20. Ban K, Wile B, Kim S, Park H-J, Byun J, Cho K-W, et al. Purification of cardiomyocytes from differentiating pluripotent stem cells using molecular beacons that target cardiomyocyte-specific mRNA. Circulation. 2013;128(17):1897-1909. doi: 10.1161/CIRCULATIONAHA.113.004228

21. Newman PJ, Newman DK. Signal Transduction Pathways Mediated by PECAM-1. Arteriosclerosis. Thromb Vascular Biol. 2003;23(6):953-964. doi: 10.1161/01.ATV.0000071347.69358.D9

22. Zeng J, Wang Y, Wei Y, Xie A, Lou Y, Zhang M. Co-culture with cardiomyocytes induces mesenchymal stem cells to differentiate into cardiomyocyte-like cells and express heart development-associated genes. Cell Res. 2008;18(S1):S62-S. doi: 10.1038/cr.2008.152

23. Petronilli V, Miotto G, Canton M, Brini M, Colonna R, Bernardi P, et al. Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. Biophys J. 1999;76(2):725-734. doi: 10.1016/S0006-3495(99)77239-5

24. Tiso N, Majetti M, Stanchi F, Rampazzo A, Zimbello R, Nava A, et al. Fine mapping and genomic structure of ACTN2, the human gene coding for the sarcomeric isoform of alpha-actinin-2, expressed in skeletal and cardiac muscle. Biochem Biophys Res Commun. 1999;265(1):256-259. doi: 10.1006/bbrc.1999.1661

25. Wade R, Eddy R, Shows TB, Kedes L. cDNA sequence, tissue-specific expression, and chromosomal mapping of the human slow-twitch skeletal muscle isoform of troponin I. Genomics. 1990;7(3):346-357. doi: 10.1016/0888-7543(90)90168-t

26. Nakao K, Minobe W, Roden R, Bristow MR, Leinwand LA. Myosin heavy chain gene expression in human heart failure. J Clin Invest. 1997;100(9):2362-2370. doi: 10.1172/JCI119776

27. Bird SD, Doevendans PA, van Rooijen MA, Brutel de la Riviere A, Hassink RJ, Passier R, et al. The human adult cardiomyocyte phenotype. Cardiovasc Res. 2003;58(2):423-434. doi: 10.1016/s0008-6363(03)00253-0

28. Bartels MC, Otten HG, Van Gelderen BE, Van der Lelij A. Influence of HLA-A, HLA-B, and HLA-DR matching on rejection of random corneal grafts using corneal tissue for retrospective DNA HLA typing. Br J Ophthalmol. 2001;85(11):1341-1346. doi: 10.1136/bjo.85.11.1341

29. Hall B, Duggin G, Philips J, Bishop GA, Horvath J, Tiller D. Increased expression of HLA-DR antigens on renal tubular cells in renal transplants: relevance to the rejection response. Lancet. 1984; 324(8397):247-251. doi: 10.1016/s0140-6736(84)90297-6

30. Badylak SF, Gilbert TW. Immune response to biologic scaffold materials. Semin Immunol. 2008; 20(2):109-116. doi: 10.1016/j.smim.2007.11.003

31. Hasegawa M, Fujimoto M, Kikuchi K, Takehara K. Elevated serum tumor necrosis factor-alpha levels in patients with systemic sclerosis: association with pulmonary fibrosis. J Rheumatol. 1997;24(4):663-665. PMID: 9101498

32. Conway DJ, Holland MJ, Bailey RL, Campbell AE, Mahdi O, Jennings R, et al. Scarring trachoma is associated with polymorphism in the tumor necrosis factor alpha (TNF-alpha) gene promoter and with elevated TNF-alpha levels in tear fluid. Infect Immun. 1997;65(3):1003-1006. doi: 10.1128/IAI.65.3.1003-1006.1997

33. Pratama G, Vaghjiani V, Tee JY, Liu YH, Chan J, Tan C, et al. Changes in culture expanded human amniotic epithelial cells: implications for potential therapeutic applications. PloS One. 2011;6(11):e26136. doi: 10.1371/journal.pone.0026136

34. Imagawa D, Millis J, Olthoff K, Derus L, Chia D, Sugich L, et al. The role of tumor necrosis factor in allograft rejection. I. Evidence that elevated levels of tumor necrosis factor-alpha predict rejection following orthotopic liver transplantation. Transplantation. 1990;50(2):219-225. doi: 10.1097/00007890-199008000-00009

35. Dupont M, Souriant S, Lugo-Villarino G, Maridonneau-Parini I, Vérollet C. Tunneling nanotubes: intimate communication between myeloid cells. Front Immunol. 2018;9:43. doi: 10.3389/fimmu.2018.00043

36. Hervé J-C, Derangeon M. Gap-junction-mediated cell-to-cell communication. Cell Tissue Res. 2013;352(1):21-31. doi: 10.1007/s00441-012-1485-6

37. Massaro MS, Pálek R, Rosendorf J, Červenková L, Liška V, Moulisová V. Decellularized xenogeneic scaffolds in transplantation and tissue engineering: Immunogenicity *versus* positive cell stimulation. Mater Sci Eng C. 2021;127:112203. doi: 10.1016/j.msec.2021.112203

38. Huettner N, Dargaville TR, Forget A. Discovering cell-adhesion peptides in tissue engineering: beyond RGD. Trends Biotechnol. 2018;36(4):372-383. doi: 10.1016/j.tibtech.2018.01.008

39. Davis RP, Casini S, van den Berg CW, Hoekstra M, Remme CA, Dambrot C, et al. Cardiomyocytes derived from pluripotent stem cells recapitulate electrophysiological characteristics of an overlap syndrome of cardiac sodium channel disease. Circulation. 2012;125(25):3079-3091. doi: 10.1161/circulationaha.111.066092

40. Miki T, Strom SC. Amnion-derived pluripotent/multipotent stem cells. Stem cell reviews. 2006;2(2):133-141. doi: 10.1007/s12015-006-0020-0

41. Fang C-H, Jin J, Joe J-H, Song Y-S, So B-I, Lim SM, et al. *In vivo* differentiation of human amniotic epithelial cells into cardiomyocyte-like cells and cell transplantation effect on myocardial infarction in rats: comparison with cord blood and adipose tissue-derived mesenchymal stem cells. Cell Transplant. 2012;21(8):1687-1696. doi: 10.3727/096368912X653039

42. Francisco JC, Cunha RC, Simeoni RB, Guarita-Souza LC, Ferreira RJ, Irioda AC, et al. Amniotic membrane as a potent source of stem cells and a matrix for engineering heart tissue. J Biomed Sci Engineering. 2013; 06(12):8. doi: 10.4236/jbise.2013.612147

43. Wang T, Xu Z, Jiang W, Ma A. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. Int J Cardiol. 2006;109(1):74-81. doi: 10.1016/j.ijcard.2005.05.072

44. Sandora N, Putra MA, Busro PW, Muttaqin C, Makdinata W, Fitria NA, et al. Preparation of cell-seeded heart patch *in vitro*; co-culture of adipose-derived mesenchymal stem cell and cardiomyocytes in amnion bilayer patch. Cardiovasc Eng Technol. 2021:1-14. doi: 10.1007/s13239-021-00565-4

45. Li X, Zhao H, Qi C, Zeng Y, Xu F, Du Y. Direct intercellular communications dominate the interaction between adipose-derived MSCs and myofibroblasts against cardiac fibrosis. Protein Cell. 2015;6(10):735-745. doi: 10.1007/s13238-015-0196-7

46. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of β-adrenergic signaling in heart failure? Circ Res. 2003;93(10):896-906. doi: 10.1161/01.RES.0000102042.83024.CA

47. Lands A, Arnold A, McAuliff JP, Luduena F, Brown JT. Differentiation of receptor systems activated by sympathomimetic amines. Nature. 1967;214(5088):597-598. doi: 10.1038/214597a0

48. White M, Roden R, Minobe W, Khan MF, Larrabee P, Wollmering M, et al. Age-related changes in beta-adrenergic neuroeffector systems in the human heart. Circulation. 1994;90(3):1225-1238. doi: 10.1161/01.CIR.90.3.1225

49. Vunjak-Novakovic G, Tandon N, Godier A, Maidhof R, Marsano A, Martens TP, et al. Challenges in cardiac tissue engineering. Tissue Eng Part B Rev. 2010;16(2):169-187. doi: 10.1089/ten.TEB.2009.0352

50. Dai R, Wang Z, Samanipour R, Koo KI, Kim K. Adipose-Derived Stem Cells for Tissue Engineering and Regenerative Medicine Applications. Stem Cells Int. 2016;2016:6737345. doi: 10.1155/2016/6737345

51. Khan M, Meduru S, Mohan IK, Kuppusamy ML, Wisel S, Kulkarni A, et al. Hyperbaric oxygenation enhances transplanted cell graft and functional recovery in the infarct heart. J Mol Cellular Cardiol. 2009;47(2):275-287. doi: 10.1016/j.yjmcc.2009.04.005

52. Qiu C, Ge Z, Cui W, Yu L, Li J. Human Amniotic Epithelial Stem Cells: A promising seed cell for clinical applications. Int J Mol Sci. 2020;21(20):7730. doi: 10.3390/ijms21207730

53. Zhang Q, Lai D. Application of human amniotic epithelial cells in regenerative medicine: a systematic review. Stem Cell Res Ther. 2020;11(1):439. doi: 10.1186/s13287-020-01951-w

54. Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling – concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. J Am Coll Cardiol. 2000;35(3):569-582. doi: 10.1016/s0735-1097(99)00630-0

Кокультивирование кардиомиоцитов и эпителиальных стволовых клеток амниона человека в амниотическом двуслойном матриксе в целях кардиомиогенеза

Мухаммад А. Путра [1], Нормалина Сандора [2], Тиас Р. Кусума [2], Нур А. Фитриа [2], Три В. Сетисна [3], Прибади В. Бусро [1], Арднансиа [1], Чайдар Муттакин [1], Вильям Макдината [1], Идрус Альви [1]

[1] Отдел торакальной и сердечно-сосудистой хирургии, факультет медицины, Индонезийский университет, Джакарта, Индонезия

[2] Индонезийский институт медицинского образования и исследований, Научный центр репродукции, бесплодия и планирования семьи, Джакарта, Индонезия

[3] Отдел сердечной хирургии взрослых, Национальный сердечно-сосудистый центр Харапан Кита, Джакарта, Индонезия

Резюме

В нашем исследовании разрабатывается система культивирования и доставки клеток для регенерации ткани миокарда в зоне инфаркта. Миокардиоциты человека (hCardio) и стволовые эпителиальные клетки амниона человека (hAESC) культивировали совместно на биологическом каркасе, образующем трехмерную (3D) матрицу в качестве возможного материала для заплаты на сердце. Настоящее исследование имело целью определение количественного соотношения миокардиоцитов и клеток амниона при их посеве для оценки лучших условий достижения кардиомиогенеза, а именно 1:5 или 1:6. Это соотношение соответствовало оптимальному числу 500000 клеток на см^2, что обеспечивает приготовление заплаты на сердце площадью 12 см^2. Это соотношение видов клеток уже сообщалось нами для кокультур стволовых клеток жировой ткани и миокардиоцитов. Миокардиоциты изолировали из операционного материала взятого от пациентов с гипертрофией правого желудочка. В изолированных клеточных популяциях показана экспрессия cTnT (10,7%), cKit/CD117 (16%), ICAM (94%) и PECAM+/VCAM- 33%. Амниотические эпителиальные стволовые клетки (hAESC) получали от доноров-женщин при кесаревом сечении. В этих клетках отмечена экспрессия TRA-1-60 (82,4%), SSEA-4 (28,2%), Oct-3/4 (2,9%), Nanog (11,4%), при отсутствии экспрессии иммунных антигенов, в т.ч., HLA-DR (0%), HLA-ABC (0,2%), а также низкий уровень экспрессии маркеров мезенхимальных стволовых клеток (MSC), т.е. CD73 (20,2%), CD90 (0,4%,) CD105 (59,2%). Эти клетки не проявляли фенотипа Lin phenotype (CD3, CD14, CD16, CD19, CD20, CD56). При обоих соотношениях клеток в культуре в матрице выделялись небольшие количества TNF-α (<1 пг/мл) на протяжении культивирования с 1-го по 8-й день. Обе группы экспериментов имели сравнимый уровень роста клеток на протяжении времени. Конфокальные изображения показывают, что клеточная популяция размножалась и мигрировала в глубину до 140 мкм после 5 сут. культивирования кА в группе 1:5, так и в режиме 1:6. При этом отмечалось соединение клеток и образование отростков, начиная с 5 сут., более заметно в опытах с соотношением 1:5. Отмечалась быстрая пролиферация и отсутствие отдельно лежащих клеток после 8 сут. культивирования. Экспрессия генов кардиомиогенеза, в т.ч. cTnT и ACTN2, в группе с соотношением 1:6 на 8 сут. была сравнима с таковой в нормальных кардиомиоцитах. Гены MHC в группе с соотношением клеток 1:6 также экспрессировались, хотя и в меньшей степени, чем в в нормальных кардиомиоцитах. Эта работа показала, что кокультивирование миокардиоцитов и амниотических стволовых клеток при соотношении 1:6 на бислое амниотических клеток может поддерживать развитие кардиомиогенеза из клеток-предшественников.

Ключевые слова

Амниотические эпителиальные клетки человека, кардиомиоциты человека, кардиомиогенез, амниотический бислой, каркас трехмерный, клеточная терапия.