



Effect of ascorbic acid on differentiation of human amniotic fluid mesenchymal stem cells into cardiomyocyte-like cells

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ABSTRACT

The aim of this study was to evaluate the efficiency of ascorbic acid (AA) on cell viability, cytotoxicity and the effects on cardiomyogenic differentiation of the human amniotic fluid mesenchymal stem cells (hAF-MSCs). The results of methylthiazole tetrazolium (MTT) assay and cell apoptosis assay indicated that after 24, 48 and 72 h of treatment, AA had no effect on cells viability and cytotoxicity. After treating the hAF-MSCs with 5-azacytidine (5-aza) and a combination of AA and 5-aza, the alamar blue cells proliferation assay showed the normal growth characteristic similar to control group. Especially, the morphological changes were observed between day 0 and day 21, and it was revealed that the hAF-MSCs exhibited myotube-like morphology after 7 days of cell culturing. Moreover, the treatment with a combination of AA and 5-aza was able to up-regulate the cardiomyogenic specific gene levels, which are known to play an important role in cardiomyogenesis. This was specifically notable with the results of immunofluorescence and immunoenzymatic staining in the AA combined with 5-aza treatment group, the highest expression of cardiomyogenic specific proteins was revealed including for GATA4, cTnT, Cx43 and Nkx2.5. It could be concluded that AA may be a good alternative cardiomyogenic inducing factor for hAF-MSCs and may open new insights into future biomedical applications for a clinically treatment.

1. Introduction

Nowadays, lifestyle is an important factor in determining an individual's health. According to the WHO, 60% of the factors related to an individual's health and quality of life are correlated to one's lifestyle [1]. Millions of people are known to have an unhealthy lifestyle. Consequently, they encounter illnesses, disabilities and even death. Globally, coronary artery disease (CAD) is a leading cause of death. CAD has also become a significant health problem among the Thai population and has led to a high mortality rate within the country [2]. Poor lifestyle choices include smoking, eating unhealthy foods and not getting enough exercise. These are all known causes of this disease [3]. The heart is incapable of regenerating sufficient cardiomyocytes to undergo extensive repair. Naturally, cardiomyocytes are terminally differentiated and stop dividing after birth [4, 5, 6]. Treatment options involve taking medicine, surgical procedures and stem cell therapy using mesenchymal stem cells (MSCs) [4]. Classical pharmacological treatment and surgical procedures may be

able to halt, but cannot reverse, the underlying disease process. Tissue engineering has emerged as an alternative cell-based approach, aiming at partial or full replacement of damaged organs with in vitro generated tissue equivalents [7]. Techniques used for cardiomyocyte tissue engineering are hindered by the difficulties of acquiring autologous cardiomyocytes, due to the limited number of possible donor sites and complexities associated with harvesting procedures and cell yields [8]. Embryonic stem cells possess enormous potential, but are also associated with many ethical and accompanying political issues [9]. Moreover, there are many problems associated with using MSCs obtained from human adult bone marrow, including pain, morbidity, low cell numbers upon harvesting [10, 11] and high levels of immunoreactivity [12]. High yields of cells and favorable growth kinetics of in vitro cultures are practical requirements for a clinically useful cell source for tissue engineering [9]. Amniotic fluid (AF) is one source of MSCs and it contains a heterogeneous cell population derived from placental membranes and fetal origins. The Amniotic fluid mesenchymal stem cells (AF-MSCs)

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obtained from amniocentesis in the second-trimester of pregnancy for prenatal diagnosis [13]. The advantageous consequence of obtaining AF-MSCs is that it is easy and presents a low risk of tumorigenicity [14]. However, the potential complications of an amniocentesis are pain from the procedure, 1.19% risk of miscarriage and risk of infection [15]. This is also an invasive procedure, with its own risks. It can come to the same conclusions as amniocentesis and is typically performed earlier in the pregnancy. Certainly, 3 AF-MSCs have a self-renewal capacity, multi-lineage differentiation [16, 17, 18] and useful in cell transplantation in immunologically matched recipients [17, 19]. 5-aza is a DNA methylation inhibitor that is mainly used in the treatment of myelodysplastic syndrome (MDS) and it can induce MSCs into cardiomyocytes. From previous study have proved that 10 μ M 5-aza induces cardiomyogenic differentiation [11, 20, 21]. At present, there are many cytokines and small molecules that have been found to modulate signaling pathways involved in cardiogenesis, and can thereby enhance the efficiency of cardiogenic differentiation [8, 22, 23]. AA or vitamin C is an organic compound. It was chosen from an extensive literature review and according to the molecular mechanisms involved in cardiogenesis. In this study, AA was used as an inducing factor for human amniotic fluid mesenchymal stem cells (hAF-MSCs) without retroviral transduction and reprogramming. Thus, the aim of this study was to evaluate the optimal dose of ascorbic acid that effects on cell viability, cytotoxicity and the differentiation potential of hAF-MSCs toward cardiomyocyte-like cells.

2. Materials and methods

2.1. Cell samples

The back-up human amniotic fluid cell (hAF cell) samples were obtained from the 16th–22 nd weeks of gestation by amniocentesis after prenatal diagnosis from the Human Genetics Laboratory, Department of Anatomy, Faculty of Medicine, Chiang Mai University. The back-up hAF cell samples displayed a normal karyotype (46, XX/46, XY). This study was approved and allowed by the Ethics Committee from the Faculty of Medicine, Chiang Mai University, 13th March 2018, NO. ANA-2561-05344.

2.2. Cell isolation

The direct adherence method was used to separate hAF-MSCs [24]. In summary, the hAF cells were centrifuged (C2 Series, Centurion Scientific Ltd, UK) at 2,035 g for 6 min at room temperature and then plated in 25 cm² flasks (Corning Incorporated, NY, USA) with expansion medium (BIOAMF-3TM Complete Medium) (Biological Industries, Kibbutz Beit Haemek, Israel) at 37 °C, 5% CO₂ and 95% humidity until the colonies of the adherent cells appeared. After that, the basal growth medium, Dulbecco's Modified Eagle Medium (DMEM)–high glucose (Gibco, USA) was replaced with a supplement of 10% fetal bovine serum (FBS) (Gibco, South America), gentamycin, Pen Strep (penicillin and streptomycin) (Gibco, USA). After 24 h, the culture media was changed in order to remove non-adherent cells. After the cells reached 80% confluence, they were sub-cultured using 0.25% trypsin-EDTA (Gibco, USA) and re-expanded in a 25 cm² flask under similar conditions. The medium was changed every 3 days and the 2nd passage of the hAF cell samples was used for all experiments.

2.3. Cell cultivation

The hAF cell samples were observed under DMi1 inverted phase contrast microscope (Leica Microsystems, USA). The cell samples in the 2nd passage were washed twice with sterile phosphate-buffered saline (PBS) (Amresco, Ohio, USA) and were trypsinized with 0.25% trypsin-EDTA. Subsequently, hAF cells were suspended in the basal growth medium (DMEM-high glucose with 10% FBS) and centrifuged at 2,035 g for 6 min at room temperature. After that, the supernatant was removed

and the hAF cells were used in the experiments.

2.4. Flow cytometry analysis

The hAF cells (3 samples) were used to determine MSCs marker expression. The hAF cells were trypsinized with 0.25% trypsin-EDTA and centrifuged at 2,035 g for 6 min at room temperature and they were then incubated with monoclonal antibodies; fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34, CD90 (Biolegend, San Diego, USA), mouse anti-human HLA-ABC (Immuno Tools GmbH, Friesoythe, Germany) and mouse anti-human fibroblast (EDM, Millipore Crop, UK), as well as phycoerythrin (PE)-conjugated mouse anti-human CD31, CD117, HLA-DR (Immuno Tools GmbH, Friesoythe, Germany), mouse anti-human CD44 (Pierce Biotechnology, Rockford, USA), mouse anti-CD45 (Biolegend, San Diego, USA) and mouse anti-human CD73 (Life Technologies, California, USA) for 1 h at 4 °C. FITC mouse isotype control and PE mouse isotype control (Biolegend, San Diego, USA) were used as negative controls. Cell fluorescence was evaluated using FACscan (Becton Dickinson, Lincoln Park, NJ) and analyzed using the CellQuest Pro 9.0 software (Becton Dickinson). The data were presented as mean $\pm$ SEM.

2.5. Alamar blue cell proliferation assay

The hAF-MSCs were used to measure living cell proliferation from the oxidation–reduction of the living cell metabolism through the conversion of resazurin (oxidised form, blue color) to resorufin (reduced form, pink color). Briefly, the hAF-MSCs (3 samples) were cultured in a 24-well culture plate at a density of 2×10^3 cells/well. They were then cultured under three different conditions (basal growth medium was used as control, 10 μ M of 5-aza and 10 μ M of 5-aza + 100 μ g/ml of AA treatment group) for 24 h. Thereafter, the medium was removed and 100 μ l of 10% alamar blue in DMEM was added. The samples were then incubated at 37 °C, 5% CO₂ with 95% humidity for 4 h. After that, the supernatant was evaluated using the colorimetric change from each well, while hAF-MSCs were continuously cultivated with basal growth medium. Absorbance measurement was done every other day until day 21 of cell-culturing using a spectrophotometer plate reader (Original Multiskan EK, Thermoscientific, UK) at 540–630 nm. The data were presented as mean $\pm$ SEM.

2.6. Cell viability

The viability of the hAF cells after AA (Sigma-Aldrich, St. Louis, MO, USA) treatment was determined by using MTT assay. The hAF cells (3 samples) were plated in triplicate in a 96-well culture plate at a density of 5×10^3 cells and then incubated at 37 °C, 5% CO₂ at 95% humidity for 24 h. After cell plating, cells were exposed with different concentrations of AA (1.5625–200 μ g/ml) [8, 25] for 24, 48 and 72 h, respectively. Then, the basal growth medium was discarded, replaced with MTT solution (0.5 mg/ml of Thiazolyl Blue Tetrazolium Bromide in DMEM) (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 4 h. After incubation,

Table 1
The primers for RT-qPCR and products size.

Gene (marker)	Primer sequence	Size (bp)
<i>GATA4</i>	F5'-CTGTGCCAACTGCCACACCA-3' R5'-GGCTGACCGAAGATGCGTAG-3'	437
<i>cTnT</i>	F5'-GGCAGCGGAAGAGGATGCTGAA-3' R5'-GAGGCACCAAGTTGGGCATGAACGA-3'	150
<i>Cx43</i>	F5'-GAATCCTGCTCCTGG-3' R5'-GATGCTGATGATGATAG-3'	380
<i>Nkx2.5</i>	F5'-CTGCCGCCGCCAACAC-3' R5'-CGCGGGTCCCTTCCCTACCA-3'	136
<i>GAPDH</i>	F5'-ATGGGGAAG GTGAAGGTCG-3' R5'-TAAAGCAGCCCTGGTGACC-3'	70

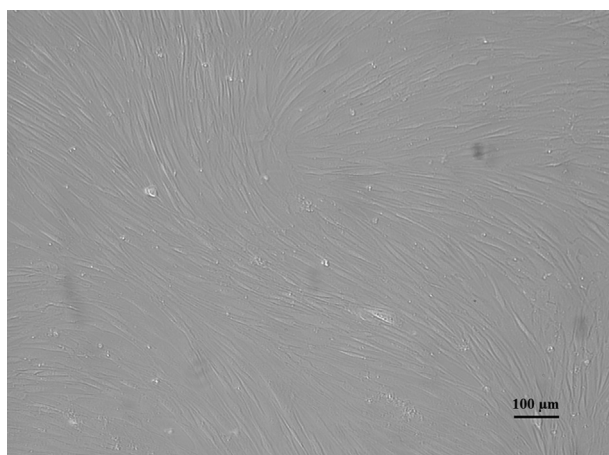


Fig. 1. The 2nd passages of hAF cells displayed fibroblast-like morphology.

MTT solution was removed and 100 μ l of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve formazan crystals. The absorbance measurement of the samples was performed using a spectrophotometer plate reader at 540 nm. The data were presented as mean $\pm$ SEM.

2.7. Cardiomyogenic specific genes expression analysis

The hAF cells (3 samples) were cultivated in a 24-well culture plate at a density of 5×10^4 cells and were incubated at 37 $^{\circ}$ C, 5% CO₂ at 95% humidity for 24 h. Afterwards, the cells were exposed to 25, 50 and 100 μ g/ml of AA for 21 days and 10 μ M of 5-azacytidine (5-aza) for 24 h. After 24 h of 5-aza treatment, the medium was changed to basal growth

medium until the 21st day. The dosages of AA in this study were of a non-cytotoxic dose and this was determined based on the results of the MTT assay. Total ribonucleic acid (RNA) was extracted by utilizing an Illutra RNAspin Mini RNA Isolation Kit (GE Healthcare, UK), and then the complementary DNA (cDNA) was synthesized from 0.5 μ g RNA using the iScript[™] cDNA Synthesis kit (Bioline, USA) according to the manufacturer's instructions. The Reverse transcriptase – quantitative PCR (RT-qPCR) was carried out using a SensiFAST[™] SYBR[®] No-ROX Kit (Bioline, USA) on a Chromo4[™] Real-Time PCR Detector (Bio-Rad, United States). The gene specific primer sequences are shown in (Table 1). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as the internal control gene for the normalization of the relative gene expression level using the $2^{-\Delta\Delta ct}$ method. The data were presented as the mean $\pm$ SEM.

2.8. Cell cytotoxicity

The cytotoxicity of the hAF cells after AA treatment was determined by using Dead Cell Apoptosis Kit with FITC annexin V and PI, for Flow Cytometry (Invitrogen, USA). The hAF cells (3 samples) were cultivated in the basal growth medium (DMEM-high glucose +10% FBS) in 25 cm² flasks at a density of 2×10^5 cells and incubated at 37 $^{\circ}$ C, 5% CO₂ at 95% humidity for 24 h. After that, cells were exposed with 100 μ g/ml of AA for 24, 48 and 72 h, respectively. For the control groups, the cells were cultured as the same condition as treatment groups without being exposed to AA. Then the cells were incubated with FITC annexin V and PI for 15 min. Finally, cell fluorescence was evaluated using BD FACS Aria[™] III and analyzed using BD FACSDiva software (BD Biosciences, USA). The data were presented as mean $\pm$ SEM.

2.9. Ascorbic treatment

After cell cultivation, the hAF cells (5 samples) were plated in a 24-

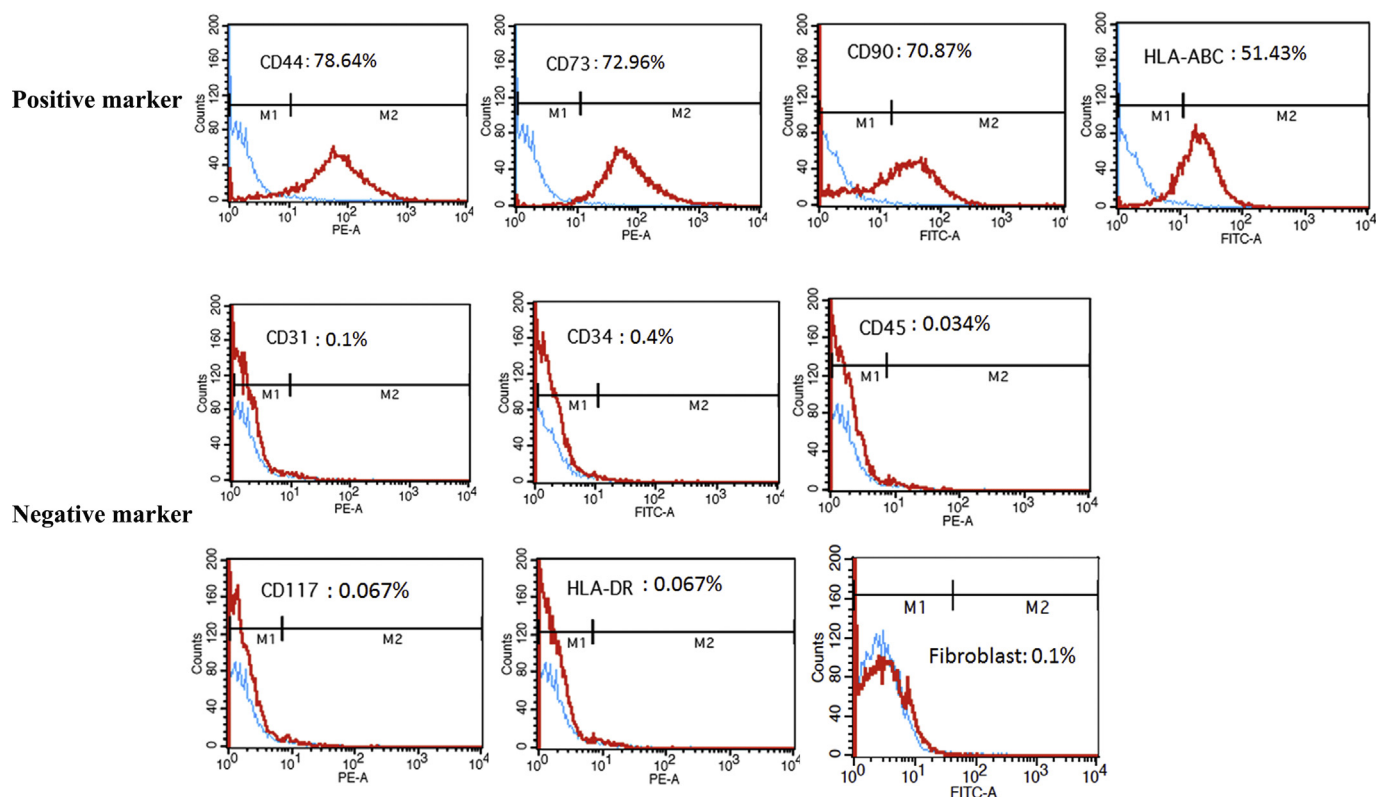


Fig. 2. Flow cytometry analysis of hAF cells to determine the MSCs characteristics showed the positive for the MSCs markers CD44, CD73, CD90 and HLA-ABC while showing negative platelet endothelial cell adhesion marker CD31, hematopoietic stem cell markers CD34 and CD45, amniotic fluid stem cells marker CD117, HLA-DR and fibroblast marker.

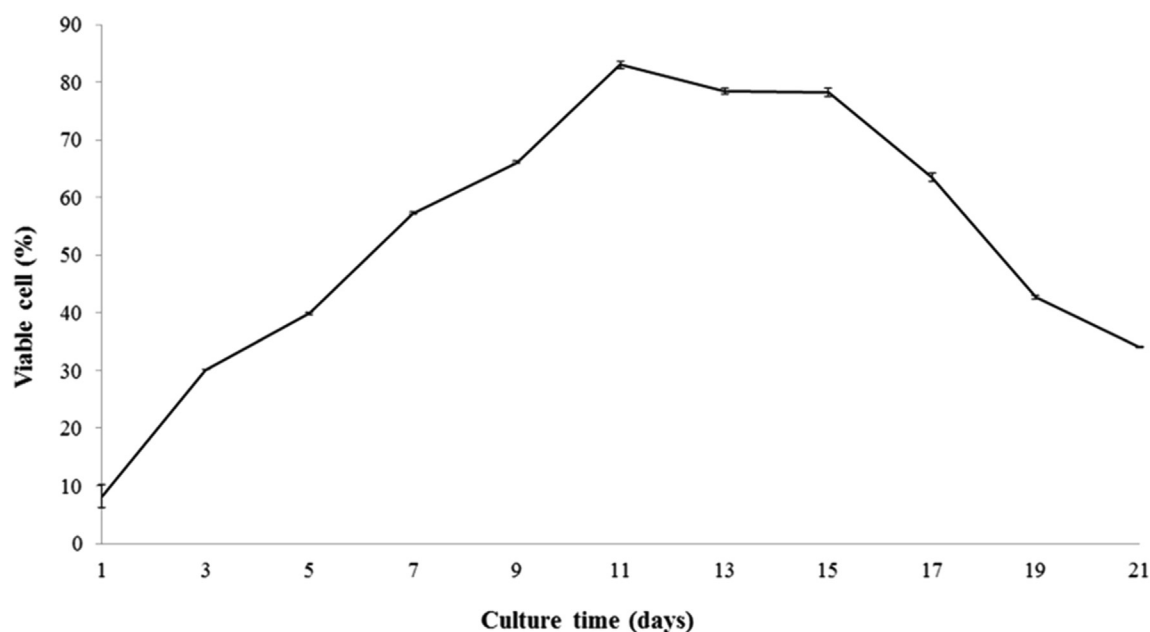


Fig. 3. Results of alamar blue assay showed the growth characteristics of hAF-MSCs culture in the basal growth medium.

well culture plate at a density of 1×10^5 cells and were incubated with basal growth medium for 24 h. After that cells were divided into three groups under different culture medium conditions. The control group was cultured with basal growth medium and the cardiomyogenic induced group was cultured with cardiomyogenic induced medium (10 μ M 5-aza) combined with or without 100 μ g/ml of AA. After 24 h, the cardiomyogenic induced medium was changed to basal growth medium and the culture medium was changed every 3 days for 21 days of cell-culturing. The alamar blue cell proliferation assay was performed every

other day until day 21 of cell-culturing. The cells morphology was observed under ImageXpress Micro 4 High-Content Imaging System. Photographs were taken with MetaXpress Software Suite (Molecular Devices, USA) beginning at day 0 until day 21. Cardiomyogenic specific gene expression was evaluated by RT-qPCR and the expression of *GAPDH* was used as the internal control gene for the normalization of the relative gene expression level using the $2^{-\Delta\Delta Ct}$ method. The data were presented as mean $\pm$ SEM. Cardiomyogenic specific protein expression was evaluated using immunofluorescence and immunoenzymatic staining.

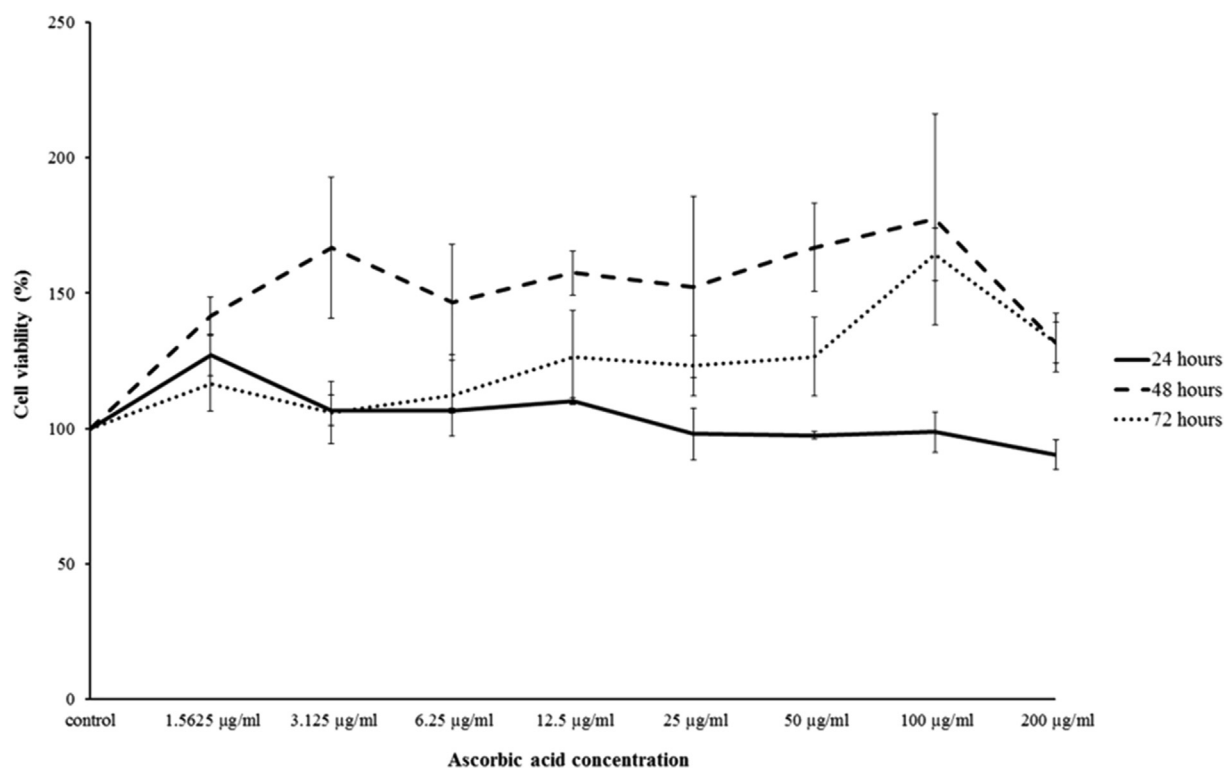


Fig. 4. The MTT assay revealed that AA had no effect on cell viability after treatment (1.5625–200 μ g/ml) for 24, 48, and 72 h.

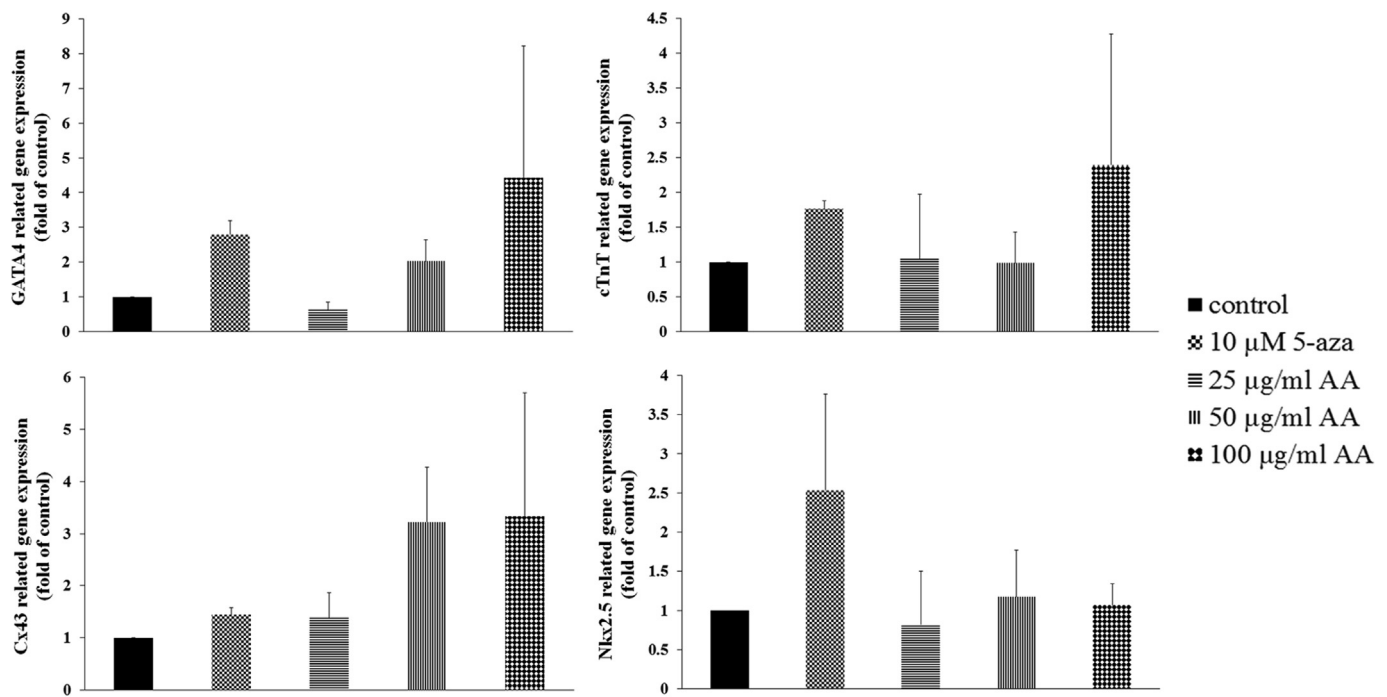


Fig. 5. AA ability to up-regulate cardiomyogenic specific genes expression. Each graph displays the expression of *GATA4*, *cTnT*, *Cx43* and *Nkx2.5*, which were normalized to *GAPDH* and were relative to the control group. Each column bar illustrates the mRNA expression level of the treated cells under basal growth medium, 10 μM 5-aza and 25, 50 and 100 μg/ml of AA respectively.

2.10. Immunofluorescence staining

The control and cardiomyogenic induced groups were cultured on coverslips (Thermo scientific, UK) for 21 days. After fixation for 30 min at 4 °C with 4% paraformaldehyde, the cell membranes were permeabilized for 5 min with 0.2% triton X-100 (Amresco, Ohio, USA) in PBS and blocked in 10% AB-serum in 1% bovine serum albumin in PBS (BSA-PBS) for 30 min at 4 °C. The cells were incubated with mouse monoclonal primary antibodies against human *GATA4*, *cTnT* and *Nkx2.5* (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. After being washed with PBS, the cells were incubated with goat anti-mouse secondary antibody conjugated with FITC (Thermo Scientific, UK) for 1 h at 37 °C. Subsequently, the nuclei and cover-slips were mounted onto the microscopic slides using anti-fade reagent with 4'-6-diamidino-2-phenylindole (Invitrogen, USA). The cells were visualized using a fluorescence microscope Olympus AX70. Photographs were taken with DP manager and DP controller (Olympus Life Science, USA). The expression of the fluorescent signal was assessed using imageJ 1.50i software and calculated by CTCTF = Integrated Density – (Area of selected cell * Mean signal of background readings). The data were presented as the mean ± SEM.

2.11. Immunoenzymatic staining

The control and cardiomyogenic induced groups were cultured on coverslips (Thermo scientific, UK) for 21 days. After fixation for 30 min at 4 °C with 4% paraformaldehyde, the cells were blocked in 10% AB-serum in 1% BSA-PBS for 30 min at 4 °C, and then incubated with mouse anti-human *Cx43* primary antibodies (Sigma-aldrich, USA) for 2 h at 4 °C. After being washed with PBS, the cells were incubated with goat anti-mouse horseradish peroxidase secondary antibody (Immuno Tools GmbH, Germany) for 1 h at 37 °C. Finally, the immunoreaction was detected by using 3, 3'-diaminobenzidine substrate (Sigma-aldrich, USA). The cells were visualized under DMi1 inverted phase contrast microscope. The signal expression was analyzed using imageJ 1.50i software and calculated by CTCTF = Integrated Density – (Area of selected cell * Mean signal of background readings). The data were presented as the

mean ± SEM.

2.12. Statistical analysis

The data were analyzed by descriptive analysis and Kruskal Wallis test following with Dunn's method were administered using SPSS version 22.0 software. A p-value of less than 0.05 was considered significantly different.

3. Results

3.1. Cell isolation and cultivation

The microscopic examination revealed that the hAF cells were adhered to culture flasks and showed a colony of heterogeneous cell population, which consisted of polygonal and fibroblast-like morphology (data not shown). In the 2nd passage, the polygonal shape seemed to disappear and the fibroblast-like morphology was identified (Fig. 1).

3.2. Flow cytometry analysis

The hAF cells in the 2nd passage positively expressed typical MSCs surface markers including CD44 (78.64 ± 5.88%), CD73 (72.96 ± 7.46%), CD90 (70.87 ± 4.24%) and HLA-ABC (51.43 ± 8.43%). Additionally, they were negatively stained for CD31 (0.1 ± 0.1%), CD34 (0.4 ± 0.06%), CD45 (0.034 ± 0.06%), CD117 (0.067 ± 0.06%), HLA-DR (0.067 ± 0.06%) and fibroblast (0.1 ± 0.1%) (Fig. 2). The data were analyzed by descriptive analysis.

3.3. Alamar blue cell proliferation analysis

The hAF cells in the 2nd passage proliferation continuously increased from the early stage of cultivation (day 1 - day 11) in the logarithmic phase. After that, the cells began the stationary phase (day 11-day 15). The highest degree of cell proliferation had been observed on day 11 with 10-fold relative to day 1. After day 15, the cell numbers began to decrease

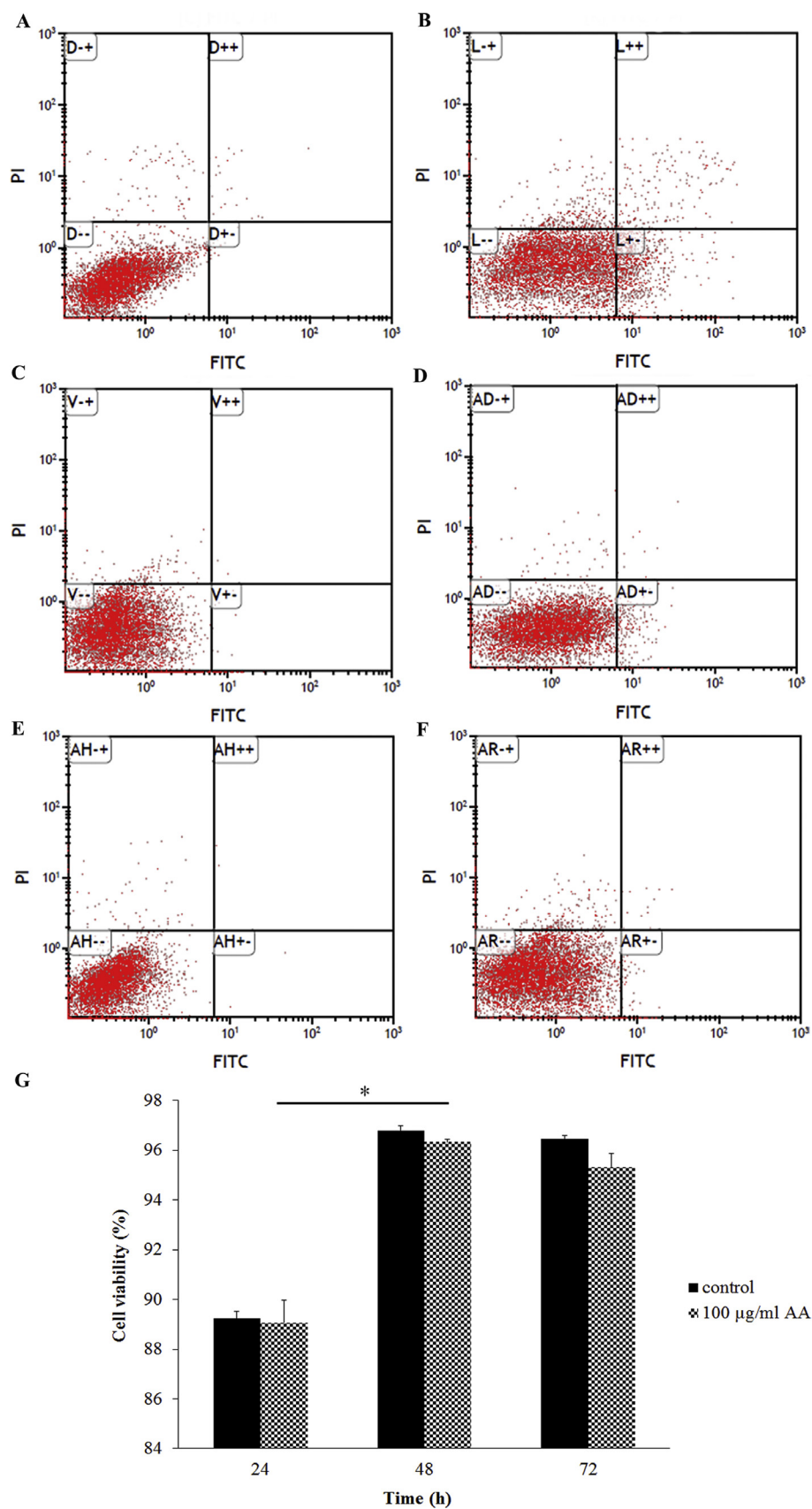


Fig. 6. Results of cell cytotoxicity using apoptotic analysis of hAF-MSCs (A,C,E) the control groups at 24 h, 48 h and 72 h respectively (B,D,F) 100 µg/ml of AA groups at 24 h, 48 h and 72 h respectively and (G) the percentage of cell viability at 24, 48 and 72 h.

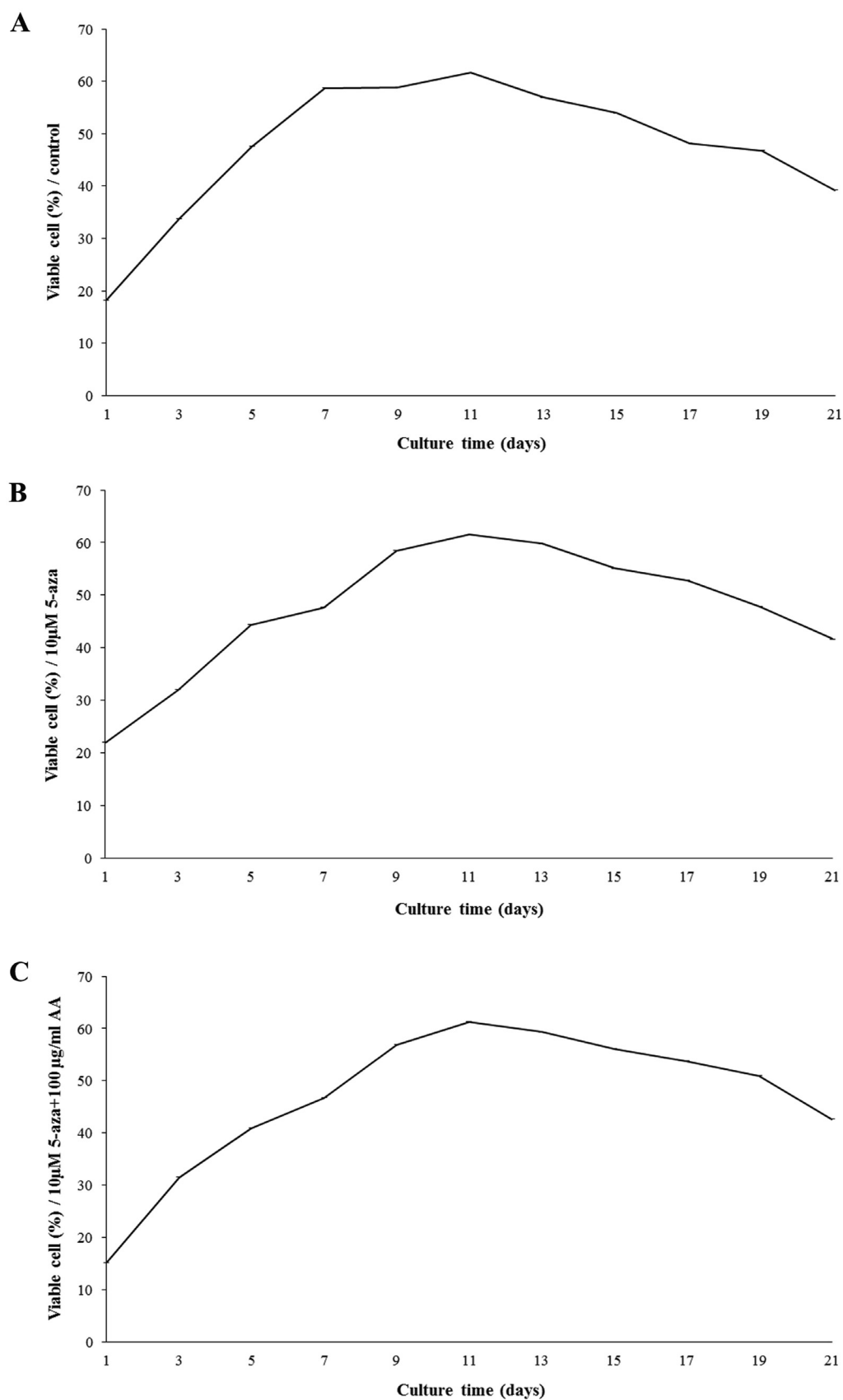


Fig. 7. Results of alamar blue assay showed the growth characteristics of hAF-MSCs after treatment (A) control group culture in the basal growth medium (B) 10 µM of 5-aza group and (C) 10 µM of 5-aza with 100 µg/ml of AA group.

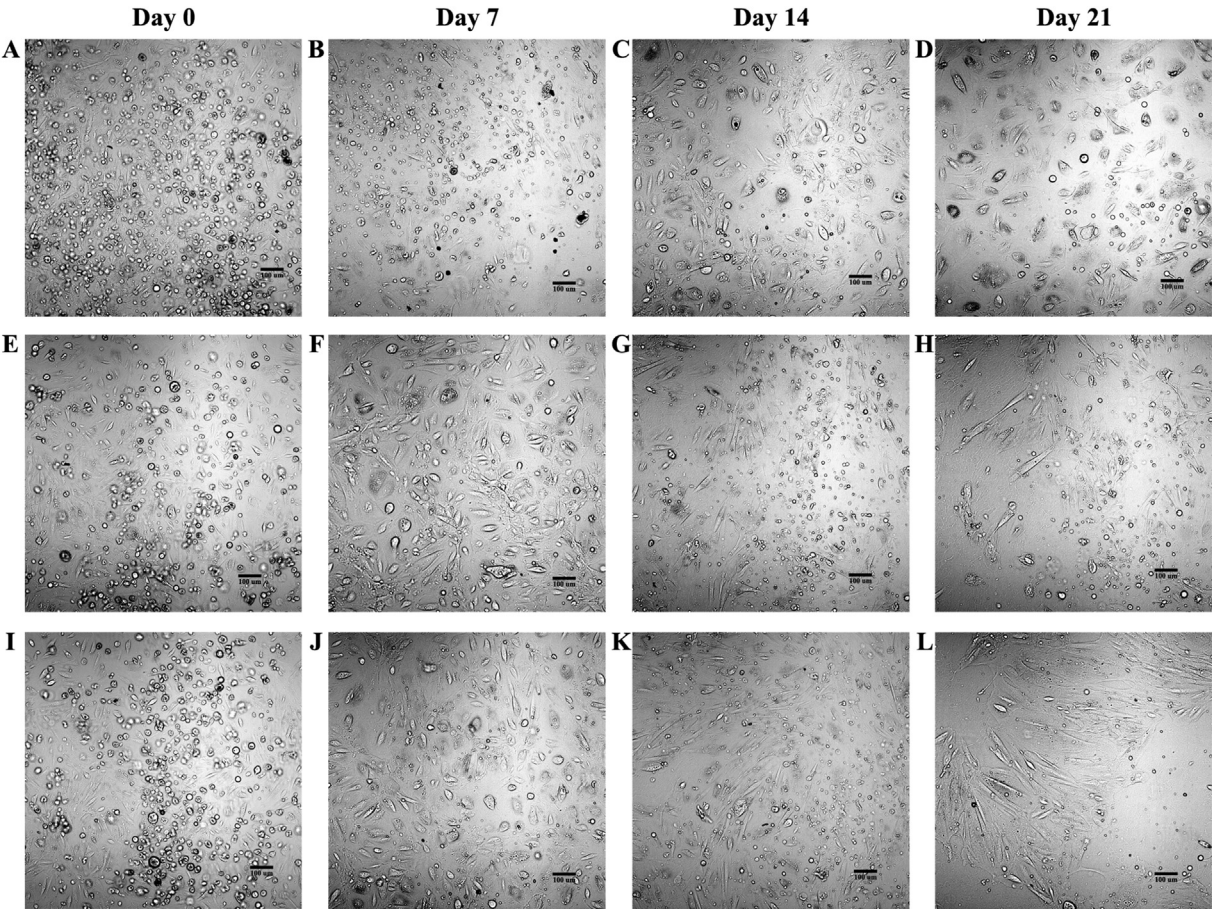


Fig. 8. Morphology of hAF-MSCs after cardiogenic induction (A–D) in control group culture in the basal medium that maintained fibroblastic morphology from day 0 to day 21 (E–H) 10 μ M of 5-aza induced group and (I–L) 10 μ M of 5-aza with 100 μ g/ml AA induced group showed elongated myotube-like morphology after 7 days until 21 days of cell culturing.

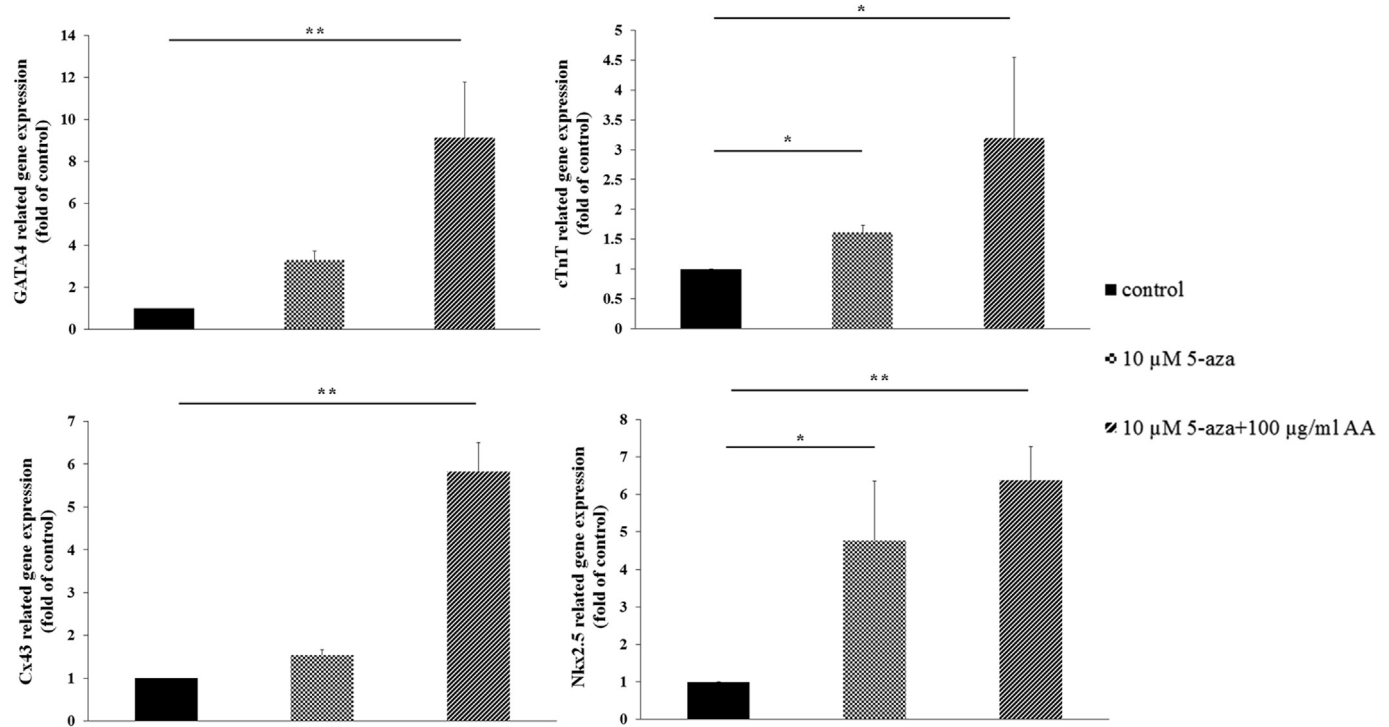


Fig. 9. Ability of AA to promote the differentiation of hAF-MSCs into cardiomyocyte-like cells. Each graph displays the expression of GATA4, cTnT, Cx43 and Nkx2.5, which were normalized to GAPDH and were relative to the control group. Each column bar illustrates the mRNA expression level of the treated cells under basal growth medium, 10 μ M of 5-aza and 100 μ g/ml of AA combined with 10 μ M of 5-aza respectively (* p < 0.05, ** p < 0.01).

(Fig. 3). The data were analyzed by descriptive analysis.

3.4. Cell viability

After the hAF-MSCs were exposed to different concentrations of AA (1.5625–200 $\mu\text{g}/\text{ml}$) for 24, 48 and 72 h, the AA treated cells showed that various concentrations had no statistically significant effect on hAF-MSCs viability (Fig. 4). The data were analyzed by Kruskal Wallis test.

3.5. Ascorbic acid-regulating effect on cardiomyogenic specific genes expression

The hAF-MSCs were investigated for cardiomyogenic specific gene expression after being exposed to AA. The results showed that at a concentration of 100 $\mu\text{g}/\text{ml}$ AA, gene levels were up-regulated through the highest expressions of GATA4 (4.43-fold), cTnT (2.4-fold), Cx43 (3.33-fold) and Nkx2.5 (1.068-fold) relative to the control. For the others, the

levels of GATA4, cTnT and Cx43 showed 1.58, 1.37 and 2.31-fold relative to the 10 μM 5-aza treatment respectively (Fig. 5). The data were analyzed by Kruskal Wallis test.

3.6. Effect of ascorbic acid on cell cytotoxicity

After the hAF-MSCs were exposed to 100 $\mu\text{g}/\text{ml}$ of AA for 24, 48 and 72 h. When compared the percentage of cell viability at each time point between AA treatment group and control group, there were not statistical different ($p > 0.05$) (Fig. 6). The data were analyzed by Kruskal Wallis test.

3.7. Effect of ascorbic acid on cardiomyogenic differentiation

In accordance with the regulating effect of AA on cardiomyogenic specific gene expression, a suitable dose (10 μM 5-aza with 100 $\mu\text{g}/\text{ml}$ AA) was used in this experiment. The alamar blue cell proliferation assay

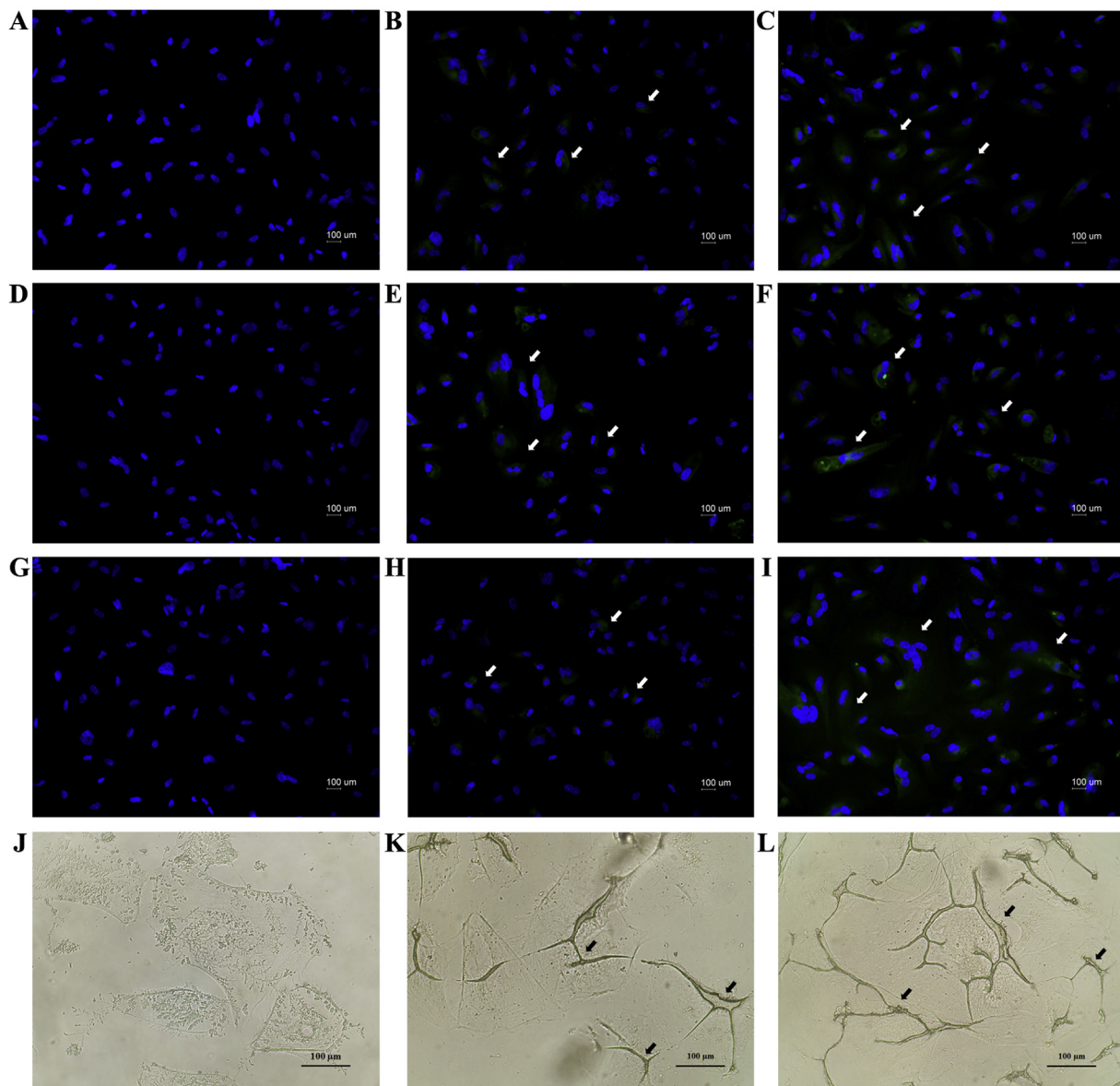


Fig. 10. Detection of the cardiomyogenic specific proteins; fluorescence staining (A–I) and immunoenzymatic staining (J–L); GATA4 staining (A) control group (B) 10 μM of 5-aza induced group (white arrow) (C) 10 μM of 5-aza with 100 $\mu\text{g}/\text{ml}$ of AA induced group (white arrow); cTnT staining (D) control group (E) 10 μM of 5-aza induced group (white arrow) (F) 10 μM of 5-aza with 100 $\mu\text{g}/\text{ml}$ of AA induced group (white arrow); Nkx2.5 staining (G) control group (H) 10 μM of 5-aza induced group (white arrow) (I) 10 μM of 5-aza with 100 $\mu\text{g}/\text{ml}$ of AA induced group (white arrow); Cx43 staining (J) control group (K) 10 μM of 5-aza induced group (black arrow) (L) 10 μM of 5-aza with 100 $\mu\text{g}/\text{ml}$ of AA induced group (black arrow).

demonstrated that cell proliferation rate in each condition (Fig. 7A-C) continuously increased from the early cultivation phase. The control group (Fig. 7A), 10 μ M 5-aza group (Fig. 7B) and 5-aza with 100 μ g/ml of AA group (Fig. 7C) slightly entered into the stationary phase without significant differences. The highest rate of cell proliferation was observed on day 11. After day 13, the cell numbers began to decrease. The morphological changes were observed at day 0 until day 21. The cells in the cardiogenic induced group cultured with 5-aza and 5-aza with 100 μ g/ml of AA showed elongated myotube-like morphology after 7 days of culturing. The myotube-like morphology appeared until 21 days of cell culture. In the control group, cultured in the basal growth medium, showed their fibroblastic-like morphology (Fig. 8). After 21 days of cell cultivation, the cells in three groups were investigated for cardiomyogenic specific genes by RT-qPCR. The results showed that at 10 μ M 5-aza with 100 μ g/ml AA treatment, gene levels could be up-regulated through the highest expressions of *GATA4* (9.13-fold), *cTnT* (3.2-fold), *Cx43* (5.82-fold) and *Nkx2.5* (6.38-fold) relative to the control. Additionally, at 10 μ M 5-aza with 100 μ g/ml AA treatment, all cardiac specific genes appeared at a higher level than 10 μ M 5-aza treatment, which were observed at 2.77-fold (*GATA4*), 2-fold (*cTnT*), 3.78-fold (*Cx43*) and 1.33-fold (*Nkx2.5*) (Fig. 9). The data were analyzed by Kruskal Wallis test following with Dunn's method. After 21 days of cell-culturing, the cells that had been cultured on coverslips in three groups were investigated for cardiomyogenic specific proteins. The results of the immunofluorescence staining and immunoenzymatic staining indicated that at 10 μ M 5-aza with 100 μ g/ml, the AA treatment group was strongly positive for cardiomyogenic specific proteins including *GATA4* (3.04-fold), *cTnT* (1.45-fold), *Cx43* (3.85-fold) and *Nkx2.5* (2.8-fold) relative to the 10 μ M 5-aza treatment, but it was not positive in the control group ($p < 0.05$) (Fig. 10) (Fig. 11). The data were analyzed by Kruskal Wallis test following with Dunn's method.

4. Discussion

Previous studies have demonstrated that AF-MSCs represent the intermediate stage between embryonic stem cells and adult stem cells [17, 26]. About 90% of the AF-MSCs express the octamer-binding

transcription factor 4 that is recognized as the transcription factor of embryonic stem cells and can express pluripotent stem cells markers; TERT [19]. Moreover, AF-MSCs are capable of self-renewal, high expansion rates, possess a high number of isolated cells [27] and can be differentiated into various cell type such as chondrocytes [28, 29], endothelial cells [30] and osteocytes [31]. In this study, the hAF cells exhibited fibroblast-like morphology adhering to the plastic culture flask in the 2nd passage, in agreement with previous studies reported that these cells can be classified into three types based on their morphological and growth characteristics: amniotic fluid (AF)-type epitheloid (E)-type and fibroblastic (F)-type cells. Fibroblast type (F-type cells) which are believed to have originated from mesenchymal tissues, easier to sub-culture, more selective and have highest growth potential [27]. It was concluded that hAF-MSCs could easily be expanded and could exhibit fibroblastoid spindle shape morphology [28, 32, 33, 34].

Several published studies have reported that hAF-MSCs positively expressed MSCs markers including CD44, CD73, CD90 and HLA-ABC [35, 36, 37], while there were no expressions of platelet endothelial cells (CD31), hematopoietic stem cells (CD34 and CD45), c-Kit (CD117), HLA-DR and fibroblast markers [27, 38]. The determination of hAF-MSCs proliferation using alamar blue cell proliferation assay exposed the continuously increased from the logarithmic phase of the cell cycle during day 1–11. After that, the highest cell proliferation had been observed on day 11, following a stationary phase of the cell cycle. Afterward, the cell numbers began to decrease. The growth characteristics of MSCs using an alamar blue cell proliferation assay indicate that these cells had a proliferation capacity, which is related to MSCs growth and proliferation properties. This finding was found to be different in AF cells that were derived from various gestational stages [29, 30, 34, 39].

From our previous study have proved that 10 μ M 5-aza induces cardiomyogenic differentiation of hAF-MSCs [34]. In this present study, AA was used as a cardiomyogenic inducing factor combined with 10 μ M 5-aza for human amniotic fluid mesenchymal stem cells (hAF-MSCs) without retroviral transduction and reprogramming. The results of this combined induction showed the highest expression of cardiomyogenic specific proteins and genes. AA or Vitamin C is commonly known to be a cofactor in many biological reactions. It is an essential nutrient. The

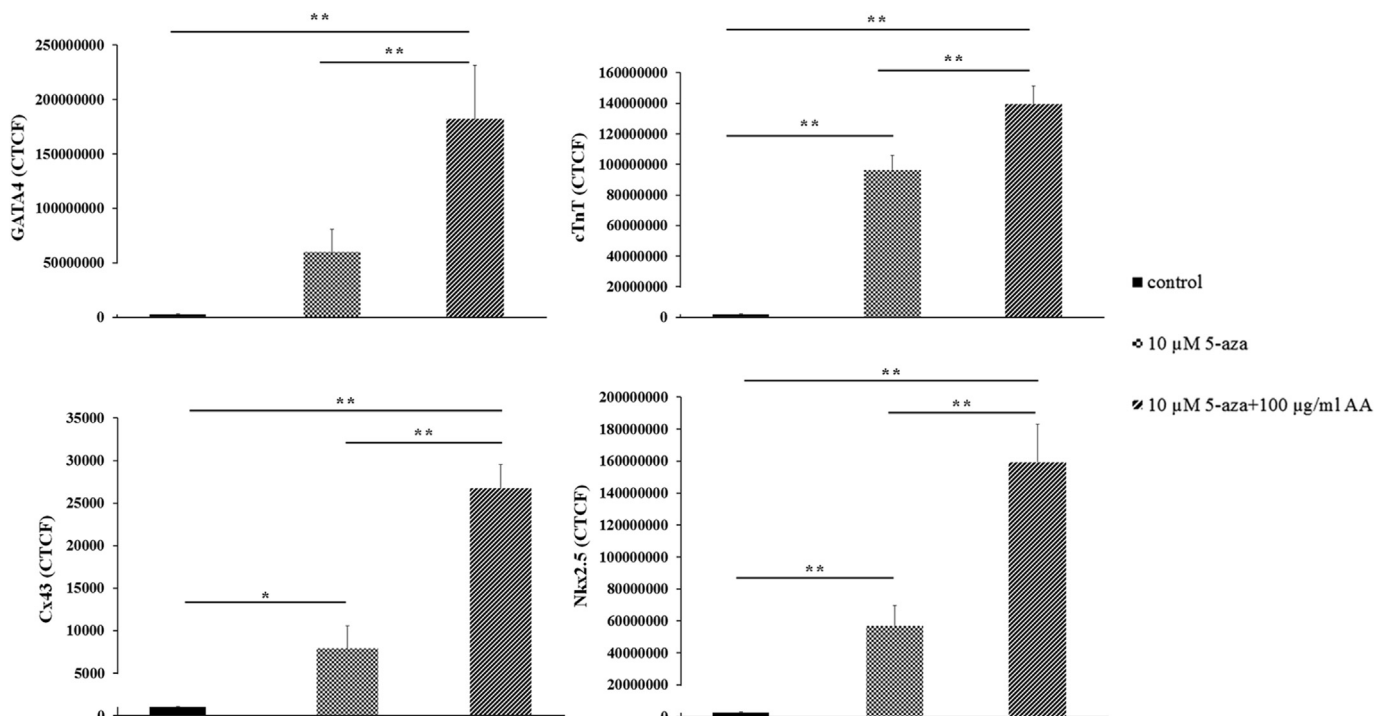


Fig. 11. Image J analysis showed the result of CTCF (the expression of GATA4, cTnT, Nkx2.5 and Cx43 proteins signal) (* $p < 0.05$, ** $p < 0.01$).

effects of AA are often attributed to its antioxidative properties [40]. Furthermore, nowadays AA has been demonstrated to promote the cardiac differentiation of embryonic stem cells [41, 42] and has been applied in several reported protocols in embryonic stem cells, induced pluripotent stem cells [25, 43] and fibroblasts [8]. Herein, our study had examined the cell viability, cytotoxicity and effectiveness of AA on the cardiomyogenic differentiation of hAF-MSCs. AA (1.5625–200 µg/ml) was not effect on hAF-MSCs viability and cytotoxicity after 24, 48 and 72 h of treatment. When treating the hAF-MSCs with a combination of 100 µg/ml AA and 10 µM 5-aza, the process was able to up-regulate cardiac specific genes and protein levels that play an important role in cardiomyogenesis. GATA4 is an important transcription factor which increases the potential of MSCs to be differentiated into cardiomyocytes [44], cTnT is a protein which functions as a part of the troponin complex of myofibrils only in the cardiac muscles [45, 46], Cx43 is one variety of the gap junction proteins [47] that maintain electrical activity [48] and Nkx2.5 is a transcription factor for cardiac development which plays an important role in early cardiac development [46]. In accordance with previous studies, we have reported that AA can enhance cardiomyogenic specific gene expression [49, 50], exert cardiogenic effects by increasing ROS levels [51] and activate the MEK-ERK1/2 pathway [25].

Accordingly, several studies have revealed that 5-aza induced the up-regulation of phosphorylated cardiomyogenic specific genes through the ERK pathway and these results suggested that the sustained activation of ERK by 5-aza contributed to the induction of the differentiation of MSCs into cardiomyocytes in vitro [52].

This study demonstrated that hAF-MSCs the characteristics of MSCs; express the MSCs positive markers, the ability of increased proliferation and could be potentially differentiated 334 into cardiomyocyte-like cells. Furthermore, the results of cell cytotoxicity demonstrated that AA had no cytotoxicity effects on hAF-MSCs and revealed some encouraging effects on cardiomyogenic differentiation. These results indicated that combined induction could improve the cardiomyogenic differentiation of hAF-MSCs. However, at present, the sample size is limited due to the source of MSCs. Therefore, further studies are required to increase sample sizes to improve the confidence intervals and significant difference in the data. Moreover, the proper use of ascorbic concentration that suitable for cardiomyogenic induction depending on individual source of MSCs. In conclusion, it has been suggested that 10 µM 5-aza with 100 µg/ml AA could be considered an effective supporting cardiomyogenic supplementary factor for cardiomyogenic differentiation in hAF-MSCs.

Declarations

Author contribution statement

Runchana Markmee: Conceived and designed the experiments; Wrote the paper.

Sirinda Aungsuchawan: Conceived and designed the experiments.

Peraphan Pothacharoen, Waleephan Tancharoen, Suteera Narakornsak, Tanongsak Laowanitwattana, Kanokkan Bumroongkit, Chani-porn Puaninta, Nathaporn Pangjaidee: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Data associated with this study is available from the authors upon reasonable request.

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