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Research Article

Adipose-Derived Mesenchymal Stem Cells Differentiation Toward Cardiomyocyte-Like Cells on the PCL/PANI Nanofibrous Scaffold: An Experimental Study

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Background: Owing to the fact that the heart tissue is not able to repair itself. Biomaterial-based scaffolds are important cues in tissue engineering (TE) applications. Recent advances in TE have led to the development of suitable scaffold architecture for various tissue defects.

Objective: Given the importance of cellular therapy, it was the aim of the present study to differentiate cardio myocyte cells from human adipose-derived mesenchymal stem cells (Ad-MSCs) using suitable induction reagents (namely, 5-azacytidine and transforming growth factor beta (TGF-β)) on poly-caprolactone (PCL)/Poly aniline (PANI) Nano fibrous scaffolds prepared by electrospinning.

Materials and Methods: For this purpose, the adipose-derived mesenchymal stem cells (Ad-MSCs) were initially isolated and characterized before cultivation on the PCL/PANI Nano fibrous scaffold to be treated for 21 days with 5-azacytidine either singly or in combination with TGF-β in medium. The scaffold's morphological and cell attachment properties were investigated using electron microscopy (SEM). Finally, the cardio myocyte differentiation of Ad-MSCs on the scaffold was studied using both quantitative Real-time PCR (qPCR) and flow-cytometry while the expression rates of the cardio myocytes' specific genes (Gata4, NKX2.5, MYH-7, and Troponin I) were also determined.

Results: The results of Ad-MSCs culture, MTT assay, and SEM indicated that the cells had well proliferated on the PCL/PANI scaffolds, showing the biocompatibility of the nanofibers for cellular growth and adhesion. After 21 days of induced cardio myocyte differentiation by both agents, Real-time PCR revealed increases in the expressions of Gata4, Troponin I, MYH-7, and NKX2.5 genes in the cells cultured on the PCL/PANI scaffolds while the flow-cytometry test approved the expression of troponin I.

Conclusion: The data obtained showed that the PCL/PANI Nano fibrous scaffolds were able to promote and support mesenchymal stem cell transformation to cardio myocyte cells. Generally speaking, the results of the study might be exploited in future *in vitro* and *in vivo* experimental model studies of cardio myocyte differentiation using co-polymer scaffolds.

Key words: Ad-MSCs, Cardio myocytes, PCL/PANI scaffolds, TGF-β1, 5-azacytidine

1. Background

Post-myocardial infarction (MI) heart failure is a clinical condition with high morbidity and mortality (1). The only therapy available for the end-stage heart failure is heart transplant while other treatment options merely slow the disease progression (2). A novel, alternative procedure to treat MI as a clinically grave condition is stem cell transplantation (3). Stem cell-based tissue

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engineering technology employed to differentiate stem cells into cardiomyocytes and to regenerate new functional myocardium seems to be a promising and practical method of treating MI (4, 5).

Mesenchymal stem cells (MSCs) reportedly offer a good choice for adult stem cell transplantation (6) due to their advantages including self-renewal, immune regulation, multi-lineage differentiation potential, hematopoietic support, and stem cell implantation (7). Adipose tissue shows some promising benefits. It can be easily collected in higher amounts and from different parts of the human body. The harvesting procedure is often part of a different operation and is not necessarily the only reason for the intervention. Most of the obtainable tissue is ethically uncontroversial as it is waste material resulting from the operation (8). Human adipose-derived stem cells (Ad-MSCs) may be a desirable candidate for cardiac tissue engineering as they can be easily extracted in large quantities (~100,000 cells per 100 mL of blood/saline collected from sonicated lipoaspirate), show high proliferation rates in culture media, and offer the capacity to differentiate into different cell types (9-11).

Low concentrations of 5-azacytidine have been selected for use in cardiomyocyte differentiation of stem cells (12). Three-dimensional (3D) tissue engineered scaffolds, especially those prepared by electrospinning of nanofibers, are porous similar to the native extracellular matrix. This resemblance results in the production of a fine pattern and environmental cue to facilitate cell growth, proliferation, differentiation, and new tissue formation (13). Poly-caprolactone (PCL) belongs to a family of poly a-hydroxyl esters that is a biocompatible and biodegradable material approved by FDA for biomedical applications while polyanilines belonging to a class of conductive polymers appear to be promising materials for the bioengineering applications due to their stability and ease of synthesis (14).

2. Objectives

The differentiation of hAd-MSCs into cardio myocyte-like cells on Nano-fibrous scaffolds has been rarely evaluated. In this study, Ad-MSCs were cultured on a co-polymer PCL/PANI Nano fibrous scaffold to investigate their capacity for differentiation into cardio myocyte-like cells in the presence of 5-azacytidine either alone or in combination with TGF-β. Finally, the differentiation of the cells was confirmed based on gene expressions on RNA and protein levels by quantitative Real-time PCR (qPCR) and flow-cytometry, respectively.

3. Materials and Methods

CD marker antibodies from Biolegend, cardiac troponin I antibody from Abcam, osteogenic and adipogenic media from stem cell research center (STRC) and other chemicals were purchased from Sigma (USA).

3.1. Fabrication of the PCL/PANI Scaffold

In this experimental study, polycaprolactone (Mw = 80,000) and PANI (Mw ~ 50,000) were used to synthesize the PCL/PANI scaffolds. Briefly, poly-caprolactone and polyaniline (80:20 w/w) were added into a solvent blend of chloroform/Dimethylformamide (DMF) (7:3, v/v) and stirred for 8h to achieve a homogenous solution of the compounds before electrospinning. Using a needle and a syringe pump, the solution thus obtained was then fed into a plastic syringe at a feeding rate of 0.4 $mL.h^{-1}$ while the voltage was set at 18 kV and a distance of 20 cm was kept between the needle tip and the collector. Finally, the PCL/PANI scaffold was exposed to O_2-plasma for 2 hours to increase its hydrophilicity and cellular attachment (15).

3.2. Characterization of the PCL/PANI Scaffold

Scanning electron microscopy (FE-SEM TESCAN MIRA3) equipped with energy dispersive X-ray detector (EDX) was employed at an accelerating voltage of 25.0 kV to study both the surface morphology, the diameter of the nanofiber scaffolds synthesized and elemental analysis. Prior to the evaluation of the micrographs, a small piece of the nanofibrous sample was sputtered with gold and finally Image J software (National Institute of Health, MD, USA) was used to calculate the average diameters of the nanofibers (16).

3.3. Fourier Transform Infrared Spectroscopy (FT-IR)

Functional groups of the PCL/Pani nanofibers scaffold were determined using Fourier Transform Infrared (FTIR) spectrometer (Thermo (AVATAR)). Dried nanofibers were mixed with KBr powder and pelletized. Measurements were taken in a range between 4000-500 cm^{-1}.

3.4. X-Ray Diffraction (XRD)

A nondestructive analysis technique to obtain information about the crystalline structure of compound is X-ray diffraction. The PCL/Pani nanofiber scaffold

was scanned by the Cu/Kα irradiations (λ= 0.1541 nm), scan speed of 2° min^{-1} and the range of 2θ between 10 and 80 ° (XRD Philips PW1730).

3.5. Cell Seeding and Proliferation on the PCL/PANI Scaffolds

Cell culture experiments were carried out using hAd-MSCs. Passage 3 Ad-MSCs were maintained in the DMEM medium supplemented with 10% FBS and antibiotics (100 $U.mL^{-1}$penicillin, 100 $\mu g.mL^{-1}$ streptomycin). Following cell confluency, the Ad-MSCs were removed from the culture dishes using 0.25% trypsin-EDTA. The cells were subsequently counted and seeded onto the PCL/PANI scaffolds on a 4-well plate at a density of 10^4 cells/well. The scaffolds were then rinsed with DPBS and fixed with 4% paraformaldehyde for 30 min.

Cell morphology was investigated 24h after cell seeding. For this purpose, the samples were naturally air-dried, coated with gold using a sputter coater, and subjected to SEM observations. Also, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) assay was employed for cell toxicity. Subsequently, the human Ad-MSCs were seeded on 5 × 5 mm PCL/PANI scaffolds at a density of $1×10^4$ cell/well in a 96-well microplate. After 2, 4, and 6 h of incubation, the scaffolds were washed with DPBS and placed in new wells. During the test, the samples were incubated for 4 h in the MTT solution before DMSO was added. Absorbance of the solution was evaluated using the ELIZA reader at 570 nm. Furthermore, cytocompatibility of the PCL/PANI nanofibrous scaffolds was investigated after 24 and 72h by seeding the hAd-MSCs onto the scaffolds with the MTT assay (17).

3.6. Isolation of Human Ad-MSCs

Adipose tissue samples were obtained from six female patients aged 30–35 years undergoing elective abdominoplasties at Erfan Niayesh Hospital under the supervision of a medical surgeon with patient consent in accordance with ethical rules. The samples were then transferred to the lab in Dulbecco's phosphate-buffered saline (DPBS) supplemented with antibiotics (100 $U.mL^{-1}$ penicillin, 100 $\mu g.mL^{-1}$ streptomycin) at 4 °C. After extensive washes with DPBS, the extracellular matrix (ECM) was enzymatically digested with 0.075% collagenase Type I in DPBS and incubated at 37 °C while being shaken vigorously for 20 min. The resulting material was washed with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and centrifuged at 1500 rpm at 4 ºC for 10 min. After the removal of the supernatant, the complete medium was added to the pellets, centrifuged at 1500 rpm at 4 ºC for 10 min, filtered through a 200 μm nylon mesh to remove fissile debris, and incubated in the control medium (DMEM-high glucose, 10% FBS, 100IU.mL^{-1} penicillin and 100 $\mu g.mL^{-1}$ streptomycin) on the culture plate at 37 °C for 48h in a humidified atmosphere containing 5% CO_2. Following incubation, the plates were washed with DPBS to remove residual non-adherent cells. The medium was changed every 3 days until the cells reached 70-80% confluence for cell passage (18).

3.7. Flow Cytometric Analysis

Untreated hAd-MSCs at passage 3 were treated with 0.25% trypsin-EDTA, extracted, and washed twice with DPBS. The cells were subsequently incubated on ice and labeled with mouse anti-human antibodies for FITC-CD45 (Cat. Num: 982316), PE-CD34 (Cat. Num: 343504), PE-CD73 (Cat. Num: 344003), and FITC-CD44 (Cat. Num: 397517). The control samples were incubated with FITC-conjugated antibodies against mouse anti-human IgG (Biolegend, USA). The labeled cells were finally subjected to flow cytometric (BD FACS Calibur (BD Biosciences, San Jose, CA, USA) FlowJo software, version 7.6.1) analysis (19).

3.8. Multi-Lineage Differentiation of Ad-MSCs

Passage 3 human Ad-MSCs in 4-well culture plates were used to induce osteocyte differentiation. For this purpose, the culture medium was changed with an osteogenic one that contained DMEM and was supplemented with 50 $\mu g.mL^{-1}$ ascorbic 2-phosphate, 10 nM dexamethasone, and 10 mM ß-glycerole phosphate. The cultures were then incubated at 37 °C under 5% CO_2 for 21 days, with the medium being replaced twice a week. Within 21 days after the cell culture, the cells were fixed in 4% paraformaldehyde and stained with 2% alizarin red S for 20 min.

For the adipocyte cells to differentiate, the proliferation medium was removed and changed with the DMEM one supplemented with FBS 10%, 50 $\mu g.mL^{-1}$ ascorbic acid 3-phosphate, 100nM dexamethasone, and 50 $\mu g.mL^{-1}$ indomethacin. After 3 weeks, the accumulated intracellular triglyceride droplets were visualized by Oil Red O staining.

3.9. Cardiomyocyte Differentiation of HAd-MSCs

Human Ad-MSCs at passage 3 were cultured in the DMEM supplemented with 10% FBS and an antibiotic solution (100 $U.mL^{-1}$ penicillin, 100 $\mu g.mL^{-1}$ streptomycin). After confluency, they were removed by treating with the 0.25% trypsin–EDTA solution. The hAd-MSCs were subsequently divided into four groups: 1) the control group receiving no supplement, 2) the Ad-MSCs cultured on PLA-PANI scaffold (designated as the PLA/PANI group), 3) the Ad-MSCs cultured on a PLA-PANI scaffold with 10 mM 5-azacytidine subsequently added for 48h (designated as the PLA/PANI + 5-azacytidine group), and 4) the Ad-MSCs cultured on a PLA-PANI scaffold with 10 mM 5-azacytidine subsequently added for 48h and 5ngr TGF-β for two weeks (designated as the PLA/PANI + 5-azacytidine + TGF-β group). Both the treated and untreated cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. In a culture medium that was changed every 48 h. The experiments were terminated after 21 days (20).

3.10. Gene Expression Analyses

Total RNA was isolated from the treated and untreated cells using Trizol. Reverse transcription reactions were performed with total RNA using the RevertAid First Strand cDNA Synthesis Kit. Real-time PCR was performed using the single-stranded cDNA sample with the SYBR Green PCR master mix. The primer sequences included (5'-3'):

Gapdh

For: CCATCACTGCCACCCAGAAGAC,

Rev: GATGACCTTGCCCACAGCCTTG;

Gata4

For: TCCAGCAACTCCAGCAACGC,

Rev: AGACATCGCACTGACTGAGAACG;

NKX2.5

For: CCGCCGCCAACAACAACTTC,

Rev: TCCCTACCAGGCTCGGATACC;

MYH7

For: CAGTGACCGTGAAGGAGGACCAG,

Rev: CGTAGATCATCCAGGAGCCGTAGC;

and Troponin I

For; CCCTCACTGACCCTCGAAACG

Rev:GGTTCCCTAGCCGCATCGC.

Each of the 40 cycles performed consisted of the following temperature program: 94 °C for 10s and 64 °C for 30 s. Relative gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method and accordingly presented. Finally, GAPDH was used as a reference gene to normalize the specific gene expression in each sample. Treatments were repeated 3 times.

3.11. Flow Cytometry

On day 21, the Ad-MSCs of the cardiomyocyte differentiated cells were detached from the PCL/PANI scaffolds using the 0.25% trypsin-EDTA; the cells were then neutralized by FBS and fixed by 4% paraformaldehyde while being maintained in a water bath at 37 °C for 15 min. The cells were subsequently permeabilized at room temperature for 5 min in an ice-cold DPBS containing 0.25% Triton-X100 and 0.01% sodium azide when they were ultimately washed twice in a DPBS containing 0.1% bovine serum albumin (BSA) and 0.01% sodium azide and incubated overnight with the primary antibody (rabbit monoclonal anti-mouse cTnI; ab47003, Abcam) at 4 °C. The cells thus treated were washed twice with 1 ml DPBS containing 0.1% BSA and 0.01% sodium azide before being incubated at room temperature in the dark place for 30 min with the goat anti-rabbit Alexa 488 (1:1000). Finally, the cells were washed twice with the DPBS containing 0.1% BSA and 0.01% sodium azide to be analyzed in the partec-Cy-Flow and FlowMax environment.

3.12. Statistical Analysis

All data are presented as mean values ± standard deviations (SD). Statistical analysis was performed using SPSS. Significant differences between groups were measured using ANOVA test, followed by Duncan test. $P<0.05$ was considered statistically significant.

4. Results

4.1. Characterization of the PCL/PANI Scaffold

The morphology of the PCL/PANI nanofibrous scaffold was investigated using SEM (**Fig. 1**) which revealed a well-fabricated nano-fibrous porous PCL/PANI matrix, similar to those of natural ECM.

Energy-dispersive X-ray (EDX) was used to analyze the chemically elemental composition of PCL/Pani nano-fibrous scaffold (**Fig. 2A**). Four signals can be observed from EDX analysis: a strong signal from C atom (91.5%) along with those from O atom (8.1%), N atom (0.1%), and Cl atom (0.3%). No visible peaks were observed for other elements or impurities.

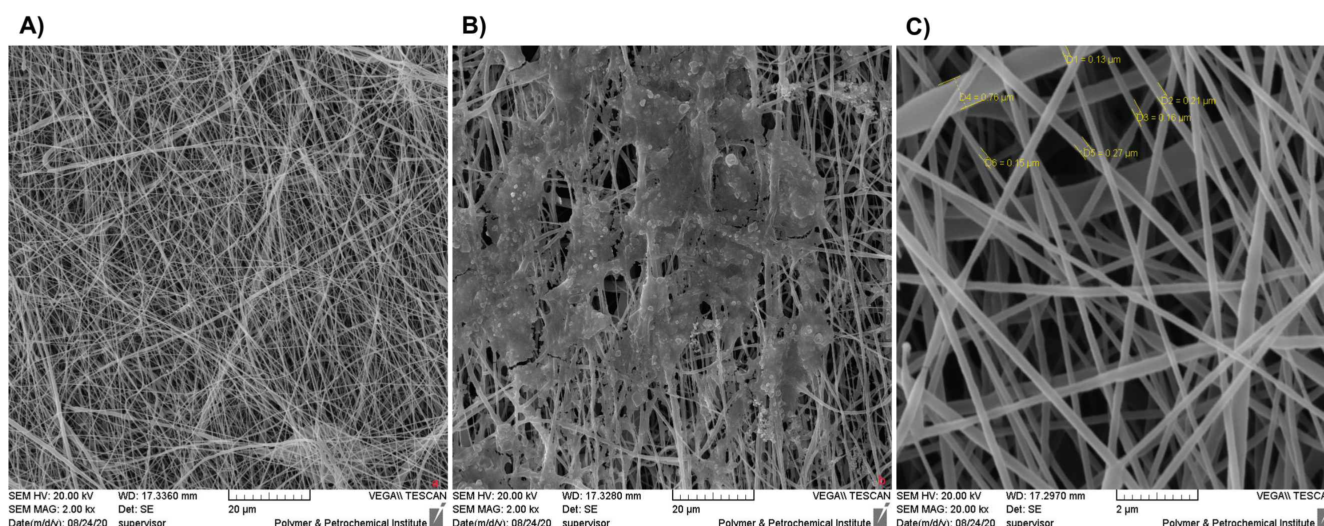

Figure 1. Scanning electron micrographs showing the PCL/PANI scaffold: **A)** without, and **B)** with cells. **C)** Fiber diameter.

X-ray diffraction (XRD) analysis revealed amplified peaks at 2θ = 44.9° and three weaker peaks at 2θ = 37.5°, 2θ=65.2 and 2θ= 78.15° (**Fig. 2B**).

Figure 2 shows the FTIR spectra of PCL/Pani. The PCL/Pani FTIR spectrum shows the characteristic bands at 1521 cm^{-1} and 1475 cm^{-1}, which are assigned to the stretching vibration of quinoid rings and the stretching mode of benzonoid rings, respectively. The bands at 1362 cm^{-1} refer to C-N stretching bonds of secondary amine group, whilst the bands at 1174 and 837 cm^{-1} are attributed to the in-plane C-H bending of quinoid structure and the out-of-plane bending of C-H bonds in the aromatic ring (**Fig. 2C**).

The Nano-fiber showed a large surface area favoring the satisfactory in-growth of cells. It was also found that the PCL/PANI nanofibrous scaffolds prepared in this study achieved a good cell adhesion after 6 hours (**Fig. 3A**). Moreover, the results of the MTT assay on the PCL/PANI scaffold were similar to those obtained for the control (**Fig. 3B**).

4.2. Characterization of Human Ad-MSCs

The morphologies of the hMSCs at passages 1 to 3 isolated from the adipose tissue showed no differences (**Fig. 4A**). More specifically, the cells showed plastic adherent properties, and fibroblast-like morphologies.

To investigate the multi-lineage differentiability of the Ad-MSCs into adipocyte and osteocyte cells, they were cultured in an adipogenic and osteogenic differentiation media for 21 days. Some of the compact and well-established areas were monitored to observe nodules in the osteogenic differentiation wells and lipid vacuoles in the adipose differentiation ones. Ad-MSCs differentiated to adipocyte and osteocyte cells after 21 days and stained positively for Alizarin Red, and Oil Red O (**Fig. 4B**).

The results of flow cytometry analysis are presented in **Figure 5**. Clearly, all the Ad-MSCs exhibited no expressions of the haematopoetic and endothelial markers CD34 and CD45 but remained immune-positive for the markers characteristic of MSCs (i.e., CD73 and CD44) (**Fig. 5**).

4.3. Cardiomyocyte Differentiation of HAd-MSCs

Quantitative Real-time PCR was used to evaluate the relative expression of cardiac specific genes in the different treatments. Addition of 5-azacytidine in combination with TGF-β to the Ad-MSCs cultured on the PCL/PANI nanofibrous scaffold was observed to lead to a significant upregulation of the mRNA gene expression of Gata4 when compared with that in the control ($P<0.05$; **Fig. 6**).

Gata4 expression in the cells differentiated on the PCL/PANI scaffolds with 5-azacytidine used singly, however, exhibited no significant difference from that observed in those treated with 5-azacytidine used in combination with TGF-β nor with those treated with no cardiomyocyte differentiation agent although Gata4 expression on the PCL/PANI scaffold treated with 5-azacytidine in combination with TGF-β showed an increasing trend.

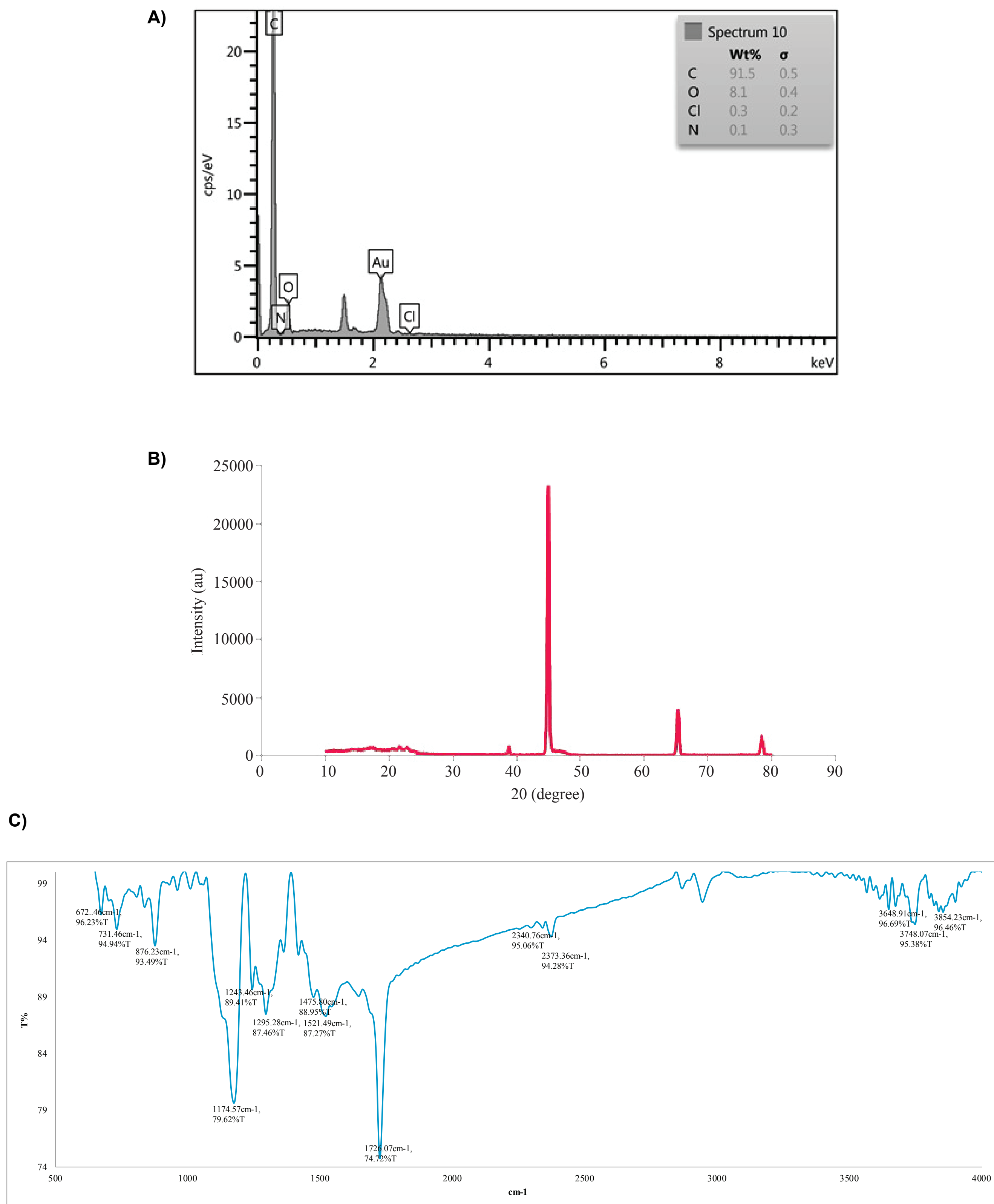

Figure 2. A) EDX of PCL/Pani nanofibrous scaffold; **B**) X-ray diffraction patterns of the fiber membranes. Notes: PCL-PAni nanofibers exhibit one strong diffraction peak at Bragg angles 2θ = 44.9° and three weaker peaks at 2θ = 37.5°, 2θ=65.2 and 2θ= 78.15°; **C**) FTIR spectra of PCL/Pani nanofibers.

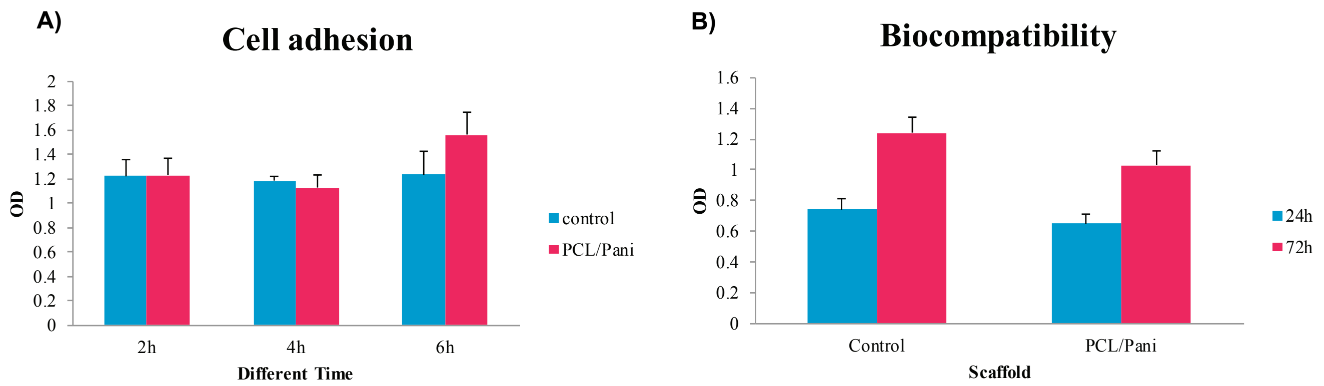

Figure 3. **A**) MTT assay results for cell attachment over different durations; **B**) Viability results of hAd-MSCs cells for 24 and 72 h by MTT assays (n=4 biological samples; mean±SD; P <0.05).

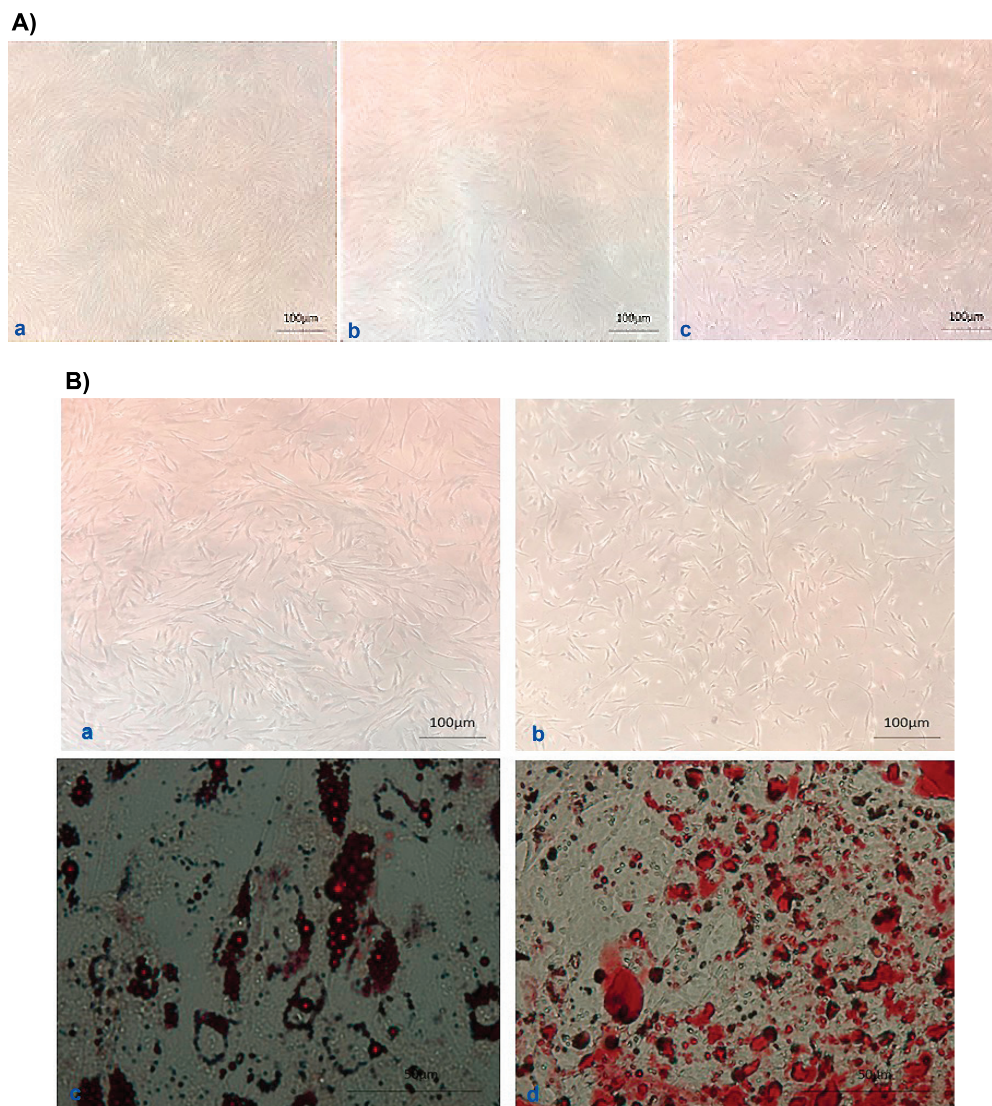

Figure 4. **A**) Human Ad-MSCs in different passages: **a**) P1, **b**) P2, and **c**) P3; **B**) Mesenchymal stem cells from human adipose tissue differentiated into adipocytes and osteocytes. Negative controls differentiated to **a**) adipocytes, and **b**) osteocytes, **c**) Alizarin Red, and **d**) Oil Red O, stain for adipose-derived mesenchymal stem cells after 21 days.

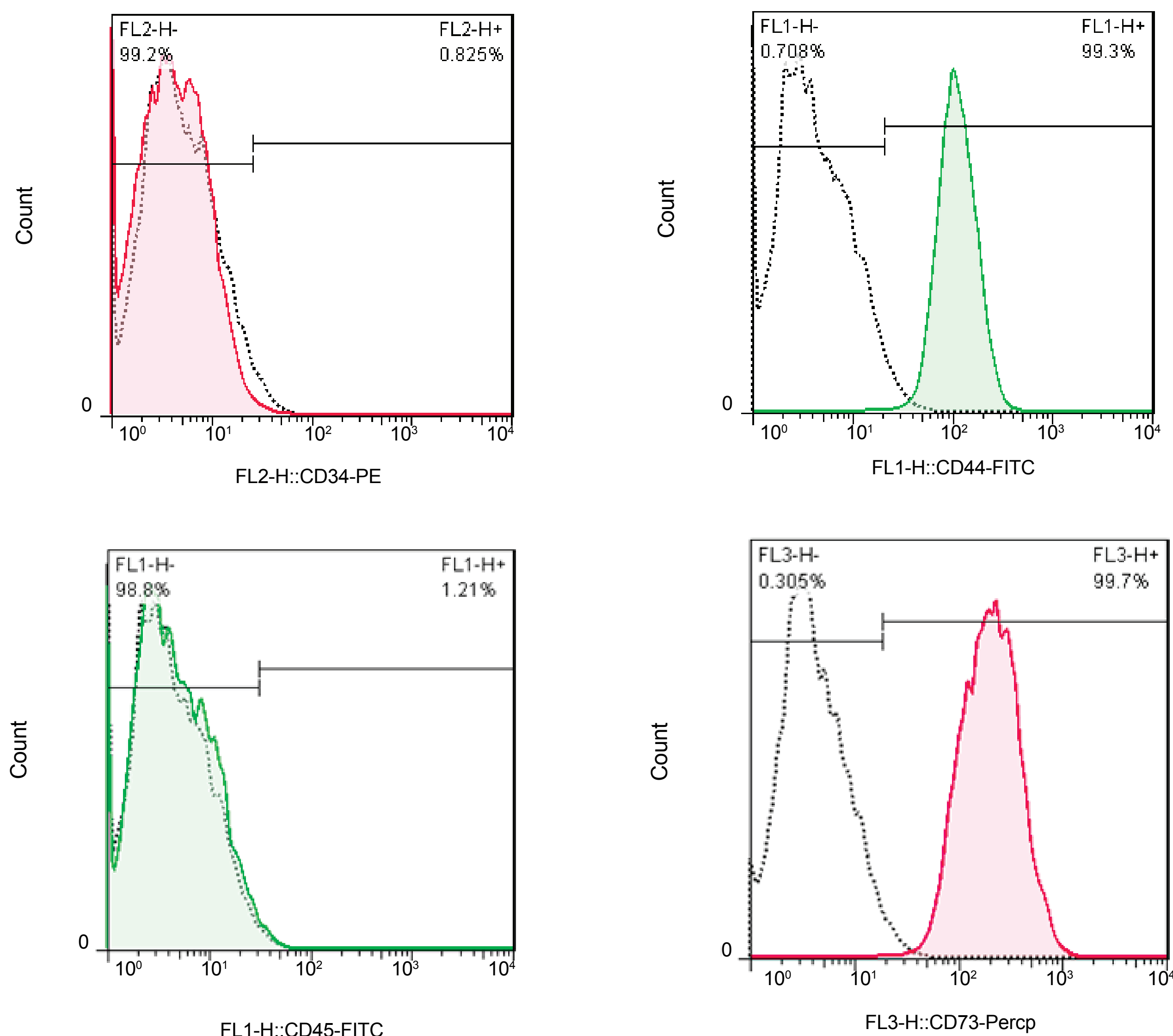

Figure 5. Identification of human adipose-derived mesenchymal stem cells (Ad-MSCs). Ad-MSCs are able to express specific surface markers CD73 and CD44 but not CD34 and CD45 (hematopoietic cell marker) as determined by flow cytometry.

Compared to the cells cultured under the other experimental conditions, the hAd-MSCs cultured on the PCL/PANI scaffolds showed significant increases ($P<0.05$) in both troponins I and MYH-7 gene expressions by day 21 in the presence of both 5-azacytidine and TGF-β, suggestive of a successful cardiomyogenic differentiation (**Fig. 6**). Finally, NKX2.5 expressions was significantly up-regulated after 21 days in the experimental groups cultured on the PCL/PANI scaffolds with 5-azacytidine used either singly or in combination with TGF-β when compared to that under observed in other treatment groups ($P<0.05$; **Fig. 6**). Further flow cytometric analysis confirmed a relatively similar level of troponin I expression in cardiomyocyte-like cells (**Fig. 7**).

5. Discussion

It was shown that hAd-MSCs can be extracted from the adipose tissue to culture the stem cells thus obtained at the desired density and selective adhesion on culture plates. The hAd-MSCs led to the expression of characteristic surface markers (namely, CD44 and CD73) while they

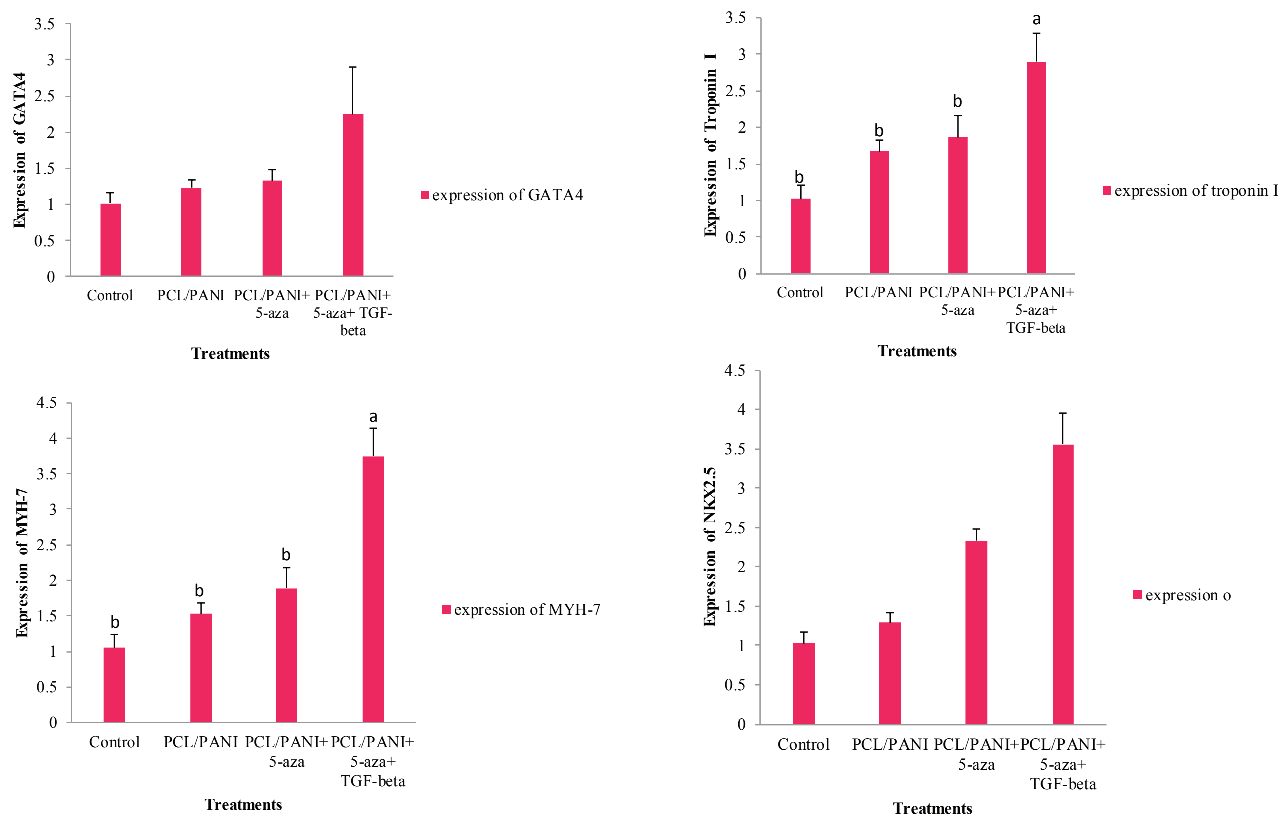

Figure 6. Quantitative evaluation of the expression of cardiomyocyte-like cells derived from hAd-MSCs seeded on the PCL/PANI scaffold after 21 days. Expressions of Gata4, MYH-7, NKX2.5 and Troponin I were investigated in human Ad-MSCs 21 days after the induction (n=4 biological samples; mean±SD). Different alphabet (**a**, **b** and **c**) on the bars indicate statistical difference (P<0.05) in gene expression.

also differentiated into adipogenic and osteogenic lineages after exposure to the specific differentiation medium.

Recently, applications of conductive polymers such as polyanilines have been extensively investigated by scaffold designs that electrically stimulate cells to regulate specific cellular activities and, ultimately, to regenerate damaged tissues.

In this line of research, scholars have combined PANI with various biological materials to achieve enhanced biocompatibility. Amirabad *et al*. (21) used polyaniline/ polyethersulfone (PANI-CPSA/PES) scaffolds with either aligned or randomly oriented nanofibers to apply electrical impulses in unidirectional and multidirectional modes. The authors found that the number of cardiac Troponin T (cTnT+) cells increased as a result of the unidirectional stimulating pulses generated by the randomly oriented fibrous scaffolds. They also observed that, compared to the cells cultured on aligned nanofibers outside a bioreactor or those cultured on randomly oriented nanofibers in a bioreactor, those cultured on aligned nanofibers in a bioreactor yielded the highest cytoskeletal fiber orientation percentage (21). It has also been demonstrated that aligned nanofiber scaffolds (PCL) increase iPSC-CPC differentiation into functional cardiomyocytes induced by Wnt inhibition (2). Moreover, PLA/PANI conductive nanofibrous sheets have been found not only to support cardiomyocyte viability, maturation, and synchronized cell beating but also to enhance cell-cell signaling (22). Culturing neonatal cardiomyocytes from Lewis rats on the PLGA/PANI co-polymer reportedly synchronized cardiomyocyte beating, implying fully developed electrical coupling between cells (23). In a different study, it was shown that the synthesis of conductive nanofibrous scaffolds with gelatin, melanin, and poly (L-lactide-co-ε-caprolactone) co-polymers used for engineering cardiac tissue improved the physiologically relevant electrical cues, resulting in increased cardiomyocyte proliferation and improved connexin-43 expression (24).

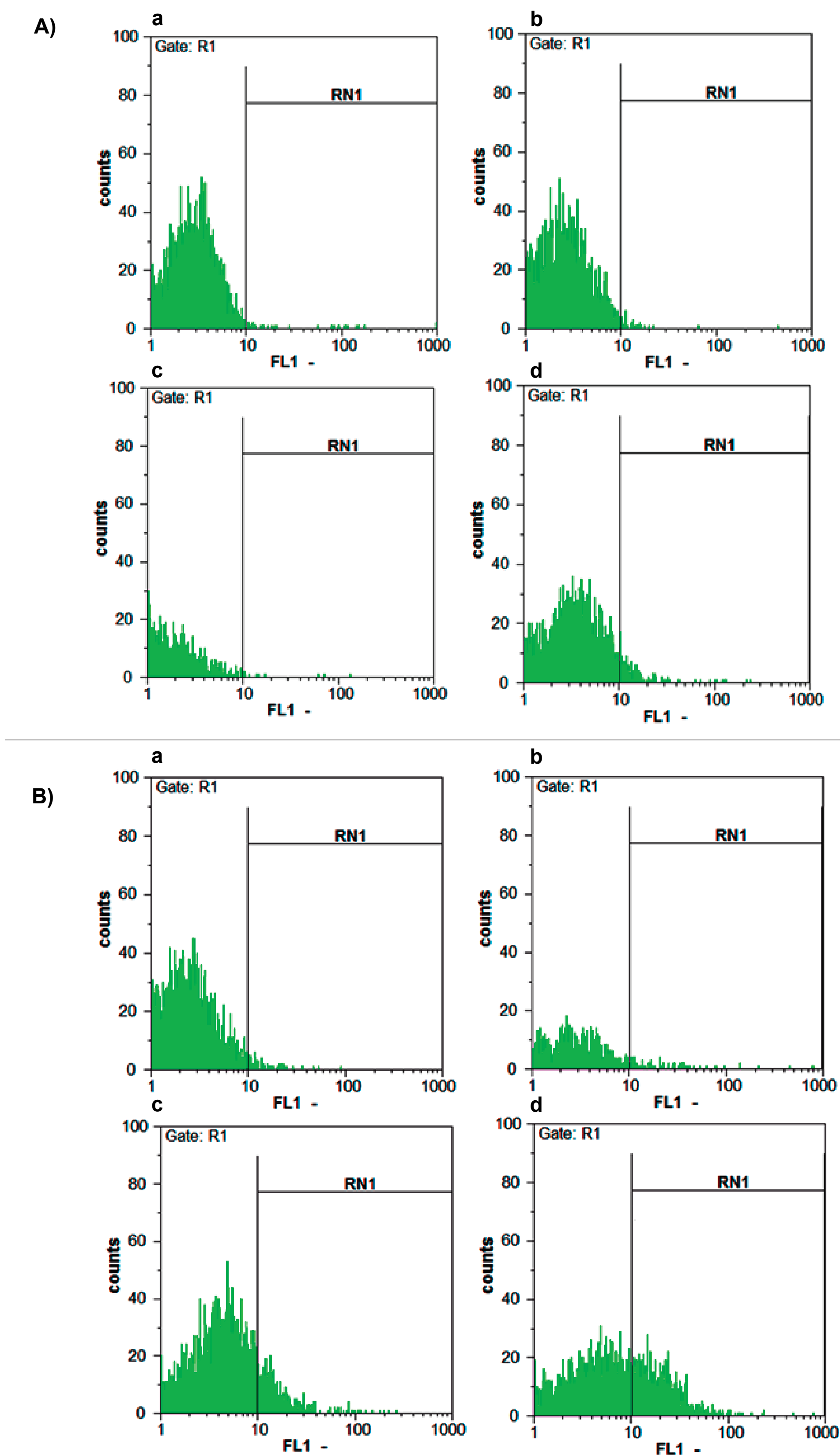

Figure 7. Analysis of cardiomyocytes specific protein Troponin I expression (by flow-cytometry) of hAd-MSCs after 21 days of cardiomyocytes differentiation. **A**) (**a**) Negative Control (NC) =0.95%; (**b**) NC: PCL/PANI= 0.98%; (**c**) NC: PCL/Pani+5-aza= 0.44%; (**d**) NC: PCL/Pani+a-zaa+TGF-β= 5.51%; **B**) (**a**) troponin I: Control= 2.02%; (**b**) troponin I: PCL/Pani =4.25%; (**c**) troponin I: PCL/Pani+5-aza =12.04%; (**d**) troponin I: PCL/Pani+5-aza+TGF-=25.42%

Also, sarcomeric α-actinin expression in hMSCs cultured on the PCL/PANI scaffold has been observed to increase as confirmed by the immunostaining test while MTT assay showed increased survival rates of the cultured cells (25). Finally, some studies have reported cell attachment supported well by PCL/PANI scaffolds or the PANI polymer combination with other polymers (22- 25).

The PCL/PANI scaffolds prepared in the present study were observed to increase the expressions of cardiac specific genes albeit with no significant differences from the results obtained from the control group. When both the cardio myocyte differentiation agents were added to the culture medium, however, the AD-MSCs were simulated to differentiate into cardio myocytes. It was, therefore, concluded that PCL/PANI nanofibrous scaffolds alone would not be a sufficient condition or would not have any influence on cardio myocyte differentiation of AD-MSCs but that this would necessarily occur in the presence of cardio myocyte induction factors including small molecules or growth factors.

It has been proved that 5-azacytidine, a DNA demethylating agent, can eliminate methyl groups from a myogenic-determinant locus in MSCs, allowing the induction of myogenic differentiation (26). Different experimental methods have shown not only that MSCs serve as cardio myocyte-like phenotypes following treatment with 5-azacytidine and hydrolyzed 5-Azacytidine but that changes in such parameters as delivery frequency, concentration, and duration of incubation affect cardio myogenic differentiation results (12, 27-29). Moreover, it has been demonstrated that 5-azacytidine functions as a potent cardio myocyte inducer during the initial phase of mouse P19 EC-cell differentiation and that its effects are brought about epigenetically and in coordination with hypo-methylation and histone acetylation-mediated hyper-expression of cardiogenesis-associated genes involving activation of ERK signaling (30). In addition, *in vitro* differentiation of human umbilical cord-derived MSCs and ovine fetal BM-MSCs into cardiomyocytes has been reportedly induced by 5-azacytidine with sustained ERK signaling at least partially contributing to this process (19, 31).

TGF-β is a protein that controls proliferation, differentiation, and some other functions in various cell types. Different cell types might generate TGF-β to control the activities of many growth factors. Moreover, the protein is capable of ameliorating the formation of connective tissues during regeneration (32). Supplementation of murine BM-MSCs with TGF-β has been reported to promote the expression of such cardiac-specific markers as cTnI, cTnT, α-MHC, and α-sarcomeric actin, suggesting that TGF-β1 might promote differentiation of BM-MSCs into cardiomocytes (33). Also, BM-MSCs treated with autologous serum and TGF-β1 have reportedly led to significant increases in the expressions of cTnT and GATA4, exhibiting not only higher proliferation rates but also an improved capacity to differentiate into cardiomyocytes (34). Treatment of MSCs with the combined 5-azacytidine and TGF-β rather than either 5-azacytidine or TGF-β alone was also shown to increase BM-MSCs differentiation into cardiomyocytes (35). This is consistent with the results of studies that showed the combined TGF-β1 and Sal-B effectively increased cardiomyogenic differentiation of BM-MSCs *in vitro* (36). The combination of low concentrations of TGF-β1 and 5-AZA used *in vitro* was reportedly able not only to improve cardiomyocyte differentiation of rat BM-MSCs possibly affected by the p-ErK1/2 mechanism but to alleviate cell damage effects as well (20).

6. Conclusion

The expression levels of cardiomyocyte specific genes revealed the capability of PCL/PANI scaffolds in enhancing hAd-MSCs differentiation into cardiomyocytes in the presence of 5-azacytidine combined with TGF-β. Moreover, hAd-MSCs differentiated on PCL/PANI were observed to provide a suitable three-dimensional substrate for the survival and growth of cardiomyocytes as a means of MI treatment.

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Ethical statement

This work was approved by National Institute of Genetic Engineering and Biotechnology (NIGEB) under Ethical No: NIGEB/93-8-7.

Authors' Contributions

Hereby we declare that all individuals contributed equally to this research in study design, data analysis, and paper drafting.

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