Original Article

Redesigning of 3-Dimensional Vascular-Muscle Structure Using ADSCs/HUVECs Co-Culture and VEGF on Engineered Skeletal Muscle ECM

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Abstract

Objective: The main objective of this study is to determine the myogenic effects of skeletal muscle extracellular matrix, vascular endothelial growth factor and human umbilical vein endothelial cells on adipose-derived stem cells to achieve a 3-dimensional engineered vascular-muscle structure.

Materials and Methods: The present experimental research was designed based on two main groups, i.e. monoculture of adipose tissue-derived stem cells (ADSCs) and co-culture of ADSCs and human umbilical vein endothelial cells (HUVECs) in a ratio of 1:1. Skeletal muscle tissue was isolated, decellularized and its surface was electrospun using polycaprolactone/gelatin parallel nanofibers and then matrix topography was evaluated through H&E, trichrome staining and SEM. The expression of *MyHC2* gene and tropomyosin protein were examined through real-time reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence, respectively. Finally, the morphology of mesenchymal and endothelial cells and their relationship with each other and with the engineered scaffold were examined by scanning electron microscopy (SEM).

Results: According to H&E and Masson's Trichrome staining, muscle tissue was completely decellularized. SEM showed parallel Polycaprolactone (PCL)/gelatin nanofibers with an average diameter of about 300 nm. The immunofluorescence proved that tropomyosin was positive in the ADSCs monoculture and the ADSCs/HUVECs co-culture in horse serum (HS) and HS/VEGF groups. There was a significant difference in the expression of the *MyHC2* gene between the ADSCs and ADSCs/HUVECs culture groups ($P<0.05$) and between the 2D and 3D models in HS/VEGF differentiation groups ($P<001$). Moreover, a significant increase existed between the HS/VEGF group and other groups in terms of endothelial cells growth and proliferation as well as their relationship with differentiated myoblasts ($P<0.05$).

Conclusion: Co-culture of ADSCs/HUVECs on the engineered cell-free muscle scaffold and the dual effects of VEGF can lead to formation of a favorable engineered vascular-muscular tissue. These engineered structures can be used as an acceptable tool for tissue implantation in muscle injuries and regeneration, especially in challenging injuries such as volumetric muscle loss, which also require vascular repair.

Keywords: Engineered Scaffold, Extracellular Matrix, Human Umbilical Vein Endothelial Cells, Mesenchymal Stem Cells, Vascular Endothelial Growth Factor

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Introduction

This experimental study continues our previous research which investigated 2D co-culture in muscle tissue challenges such as volumetric muscle loss (VML) (1). VML refers to the special volumetric, contractive, and non-returnable injury of the muscle tissue that can arise due to various causes such as severe trauma, cancer, surgery, and accident (2). Skeletal muscle, like other tissues, has a significant regenerative ability following injury; a process in which a part of the muscle can grow and develop (3). However, this regenerative response is ineffective when a large volume of the muscle is lost. According to recent studies, there is a limited treatment options for VML and no favorable, effective, and definitive treatment is found for this challenge so far (4). New methods, such as regenerative medicine with its high potential for regeneration and substitution of damaged tissues and organs, have attracted the attention of researchers in recent years (5).

In vitro designing of favorable tissue models for use in tissue engineering is considered an acceptable therapeutic option for important challenges such as VML (6). The use of stem cells in research has received attention in the last two decades. Adipose tissue-derived stem cells (ADSCs) are one of the special stem cells that have gradually found their place in these studies. They are ideal options in regenerative medicine as their favorable features such as easy isolation and culture and their control, high growth and differentiation potential, non-invasiveness, and lack of ethical consideration have attracted the attention of most researchers (7). It has been founded that decellularization is as an effective strategy in tissue

engineering, and decellularized tissues have been used in recent years in biomedical applications such as research on physicochemical properties of the extracellular matrix (ECM) and providing a special tissue scaffold with native mixture, consisting of tissue-specific macromolecules within a 3D structure, for redesigning the functional tissues of the body (8). ECM is a complex of proteins and polysaccharides as well as a combination of tissue- or organ-specific scaffold basic components. In the skeletal muscle tissue, the large part of the ECM consists of collagen fibers, different types of ECM components such as laminin, elastin, and glycosaminoglycans, growth factors, and a high number of proteoglycans and glycoproteins that are necessary for cell adhesion, permeability, migration, and differentiation (9).

Numerous studies have shown that in muscular tissue engineering, homologue tissues play a key role in the growth, differentiation, and regeneration processes of damaged tissues (10).

On the other hand, various studies have reported that differentiation of stem cells to myoblasts and formation of muscle fibers requires scaffolds with parallel and unidirectional fibers. Due to lack of certain superficial direction in biological scaffolds such as muscle ECM, the use of parallel electrospun nanofibers has gained a particular importance as an acceptable strategy for induction and improvement of myogenesis.

Numerous studies have shown that the combination of the synthetic and natural polymers of Polycaprolactone (PCL) and gelatin has a crucial role in adhesion, growth, and organization of ADSCs (11), tendon engineering advancement (12), regeneration of cardiac muscle tissue, and proliferation and differentiation of myoblasts to skeletal muscle myotubes (13).

Despite all mentioned issues, creation of an ideal engineered structure for differentiation and formation of parallel muscular myofibrils has been always associated with development of neurovascular structures as an inseparable part of normal tissues and a challenge against muscular tissue engineering. Co-culture of endothelial cells with other cell types has been used in recent years for formation of vascular structures in engineered structures as a novel method and trustable strategy to solve this challenge. For instance, it was shown that co-culture of endothelial cells with primary osteoblasts, fibroblasts or smooth muscle cells can significantly improve vascular bifurcations and buds *in vitro* (14). In addition, as the third factor in muscle tissue engineering, it seems necessary to use factors which, besides formation of vascular structures, can affect the differentiation of myoblasts. Vascular endothelial growth factor (VEGF) is a key angiogenesis regulating factor during embryogenesis that can also affect many adult cells. Studies have shown that this factor has inductive roles in endothelial cells, myoblasts, hepatocytes, and neurons in cellular migration, cellular protection against apoptosis, and induction of myoblasts for formation of myofibrils (15).

Given the existing challenge, the main objective of this experimental study is to evaluate the myogenic effects of skeletal muscle ECM, VEGF and human umbilical vein endothelial cells (HUVECs) on adipose-derived stem cells (ADSCs) to achieve a 3-dimensional engineered vascular-muscle structure.

Materials and Methods

ADSCs isolation and culture

In this experimental research, ADSCs were isolated according to a previous study (1). Briefly, the adipose tissue was removed from the gonadal region of Wistar rats, washed with PBS containing 1% penicillin/streptomycin, and fragmented to facilitate the enzymatic digestion. The specimens were incubated with collagenase type I at 37°C for 30-40 minutes and then the enzyme effect was neutralized using the culture medium [Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS)]. (DMEM, 10% FBS). To separate adipocytes from the stromal vascular fraction (SVF), the cell suspension was centrifuged at 2000 rpm for 5 minutes. The supernatant was then discarded and the cellular palate was immersed in a culture medium containing DMEM, 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. The cells were plated at 2.5×10^4 cells/cm^2 per 25 cm^2 cell culture flasks and incubated at 37˚C in 5% CO_2. After 2 days, the non-adherent cells were discarded, and the cellular passage stages were performed 4 times on adheerent cells. After 4 passages, for ADSCs characterization, the surface antigens of ADSCs including CD73, CD44, and CD90 as positive markers and CD45 as the negative marker were evaluated through flow cytometry assay. To induce myogenesis of the mesenchymal cells, ADSCs were cultured in the DMEM culture medium containing 10% FBS and 3 M 5-Azacytidine (Sigma, NY, USA) for 24 hours and then in the DMEM supplemented with 5% horse serum (HS, Gibco, NY, USA) for 7 days. Supplemented media was replaced every 48 hours.

Decellularization of skeletal muscle

Muscle tissue decellularization was performed according to previous protocols, as shown in Figure 1. After euthanization of Wistar rats, the anterior abdominal muscles (external and internal oblique) were removed. To further improve the quality of the decellularization process, the muscle layers were separated. Samples were washed in phosphate-buffered saline (PBS) with 1% antifungal and antibiotic solution, and then the muscle tissue was purified from all vascular, fat and connective tissue and washed out for 1 hour in deionised water. These samples were then treated with 0.5 M NaCl for 4 hours, followed by 1 M NaCl for 4 hours, and washed in ultra-pure water for 48 hours. After being treated with 0.25% trypsin/EDTA at 37°C for 2 hours, the samples were washed in dhen samples were processed with DNAase at 37°C for 3 hours, washed in ultra-pure water for 2 days,

rinsed in PBS for 24 hours and finally kept at 4°C till use (Fig.2A).

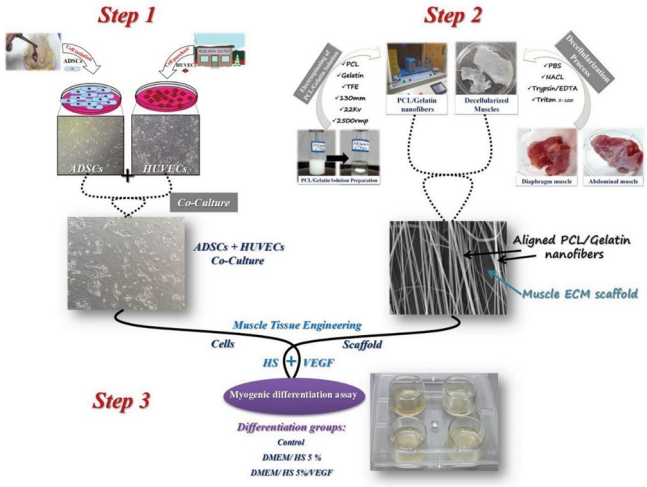


Fig.1: Schematic diagram representing the different steps of this study. Step 1; Monoculture and co-culture of stem cells and endothelial cells. Step 2; Decellularization of the muscle tissue according to the protocol (briefly) and electrospinning of the PCL/gelatin parallel nanofibers on the decellularized muscle scaffold. Step 3; Represents triad of muscle tissue engineering including scaffold (engineered ECM), cells (ADSCs/HUVECs co-culture) and factors (HS and VEGF) for evaluation of myogenic differentiation in 3 experimental groups. PCL; Polycaprolactone, ADSCs; Adipose-derived stem cells, HUVECs; Human umbilical vein endothelial cells, ECM; Extracellular matrix, HS; Horse serum, and VEGF; Vascular endothelial growth factor

Evaluation of muscle decellularization

Immediately after the muscle cell removal process, the accuracy of decellularized muscles were determined by visual examination, tissue staining and also by scanning electron microscopy (SEM) (Fig.2B). For tissue staining, a part of the prepared tissue was fixed in 10% formalin. Samples were then embedded in paraffin to provide microscopic sections and then stained with H&E and Masson's Trichrome at different magnifications.

Scanning electron microscopy

SEM evaluation was done based on previous protocols (19). First the scaffolds were washed three times with PBS and then fixed with 2.5% glutaraldehyde solution (Sigma, USA) for 30 minutes. The samples were dehydrated in different percentages of ethanol aqueous solutions from 30% to 100% successively for 10 minutes each and dried at room temperature under sterile conditions under the hood. After preparing the samples, SEM (SEM, Zeiss Evo 50, Germany) examination was used to determine the surface topography (with and without PCL/Gelatin nanofibers), arrangement of ECM fibers and structure and porosity of decellularized muscle tissue.

In addition, after fabrication of parallel PCL/Gelatin nanofibers on the ECM surface and cell culture, the samples were prepared in the same way in order to visualize the orientation and diameter of the nanofibers, surface topography of the electrospun nanofibers, cell morphology, attachment and interaction with other cells by SEM. Fiber diameters and size distribution were measured from the SEM images using Image J software version 1.46 (NIH, MD).

Fabrication of of polycaprolactone/gelatin nanofibers

PCL (Sigma-Aldrich, USA, Mw: 80000 g/mol) and gelatin type A (from porcine skin in powder form, Sigma-Aldrich, USA) scaffold were prepared by the electrospinning method as described previously (16). Briefly, to prepare a 10% (w/v) concentration solution, PCL, and gelatin in a 50/50 (w/w) ratio was dissolved separately in 2, 2, 2-trifluoroethanol (TFE, Sigma-Aldrich, USA) and stirred overnight until the mixture was dissolved completely at room temperature. Before stirring, 1% acetic acid (Fisher Scientific, USA) was added to the final solution to improve miscibility.

For the electrospinning process, PCL/gelatin solution was loaded into a 10-mL syringe with a 21-Gauge needle that was located at a distance of 130 mm from the collector. The solution flow rate, applied voltage, collector rotation speed and duration were set to 1 mL/hour, 22 kV, 2,500 rmp and 4 minutes, respectively. Moreover, before the electrospinning process, decellularized muscle ECM was placed on the collector (Fig.1). The process was carried out at room temperature within a range of relative humidity (45-50%). The PCL/Gelatin solvent turned to nanofibers and was collected in parallel orientation on decellularized muscle ECM on the collector. Before using the engineered scaffolds, in order to dry and stabilize the nanofibers, they were kept in a sterile environment at room temperature overnight.

Mechanical tensile test

The tensile strength of the decellularized muscle scaffolds, with or without parallel PCL/gelatin electrospun nanofibers, was evaluated in dry and wet conditions (Cultivated for 10 days in DMEM culture medium) by the material test device (Wance, China) equipped with a 5 kN load cell. Initially, strip-shaped pieces were prepared at a 12 mm width×30 mm length from both types of scaffolds. Tensile tests and their analysis were performed after setting the crosshead speed at 10 mm/minutes. At least three samples were investigated. Finally, using the tensile stress curve obtained from the test device, we examined the tensile strength and scaffold elastic modulus (17).

MTT assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide) assays were used to evaluate the effects of engineered muscle scaffold on ADSCs/HUVECs co-culture viability and proliferation. Briefly, ADSC and HUVEC cells were seeded in 96-well plates (1×10^4 cells/well) and were then maintained with culture media for 48 h. Then the cells were further incubated with MTT (0.5 mg/mL) for 4 hours at 37°C. After removal of the super-

natants, 700 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan product. Then using a microplate reader (BioRad, Hercules, CA) absorbance was measured at 540 nm. Optical density values of the control cells were calculated as 100% viability. Because absorbance is in proportion to the number of living cells in a sample, the MTT assay reflects the extent of cell proliferation (17).

Co-culture models

The HUVECs were obtained from the Iranian Biological Resource Center and cultured in their special culture medium (1). To track HUVECs in co-culture with ADSCs, they were labeled with C7000 according to the cell tracking guideline CellTrackerTM CM-DiI (C7000) (Sigma-Aldrich, USA). Briefly, HUVEC cells were plated at 2.5×10^4 cells/cm^2 per 25 cm^2 cell culture flasks. Then 15 µl of CM-DiI (C7000) was added for every 4 ml of culture medium and incubated at 37˚C in 5% CO_2 for 24 to 48 hours. After removal the supernatants, the attached cells could be seen in red light using fluorescence microscopy. The cell tracker was still detectable up to 2 passages.

Similar to our previous study, 2 experimental groups were designed in this research, so that each group was evaluated in 3 different culture media including the control group, the horse serum (HS) differentiation culture medium group, and the HS/VEGF differentiation culture medium group. Table S1 represents a brief description of the experimental groups (See Supplementary Online Information at www.celljournal.org).

Co-culture of HUVECs and mesenchymal cells was performed at a ratio of 1:1. The dose of VEGF used in the experimental groups was 50 ng/µL. The main points of this study are shown in Figure 1.

Cell labeling

HUVECs were marked with CM-DiI (Invitrogen, USA) according to manufacturer's instructions and then confirmed by fluorescence microscopy after 1 day.

Real-time reverse transcriptase-polymerase chain reaction analysis

Immediately after completion of the experiment on day 7, real-time reverse transcription polymerase chain reaction (RT-PCR) was used to confirm the expression of *MyHC2* according to the manufacturer's instructions. At first, for RNA extraction, cultured cells were lysed using the RNeasy plus Mini Kit (Qiagen, Gaithersburg, MD, USA). Then, by using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) the cells were quantified. cDNA synthesis using a QuantiTect Reverse Transcription Kit (Qiagen, Gaithersburg, MD, USA) was performed. The following primer sequences for amplification were used:

MyHC2-

F: 5′-GGCTGGCTGGACAAGAACA-3′
R: 5′-CCACCACTACTTGCCTCTGC-3′

GAPDH-

F: 5′-TGCTGGTGCTGAGTATGTCGTG-3′
R: 5′-CGGAGATGATGACCCTTTTGG-3′

Immunofluorescence analysis

Immediately after conclusion of the experiment on day 7, the scaffolds were washed three times with PBS and then fixed with paraformaldehyde 4% for 20 minutes. Then, the scaffolds with their surface cells became permeable using Triton X-100 for 10 minutes and were rinsed again with PBS. To prevent any non-specific adherence, they were impregnated in BSA 3% for 2 hours. Experimental groups were stained with primary antibody against anti-tropomyosin antibody (1:100, Sigma, USA) overnight at 4˚C. Then, the cultured cells on muscle scaffold were rinsed with PBS and incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:150, Sigma, USA) for 1 hour. Using 4′, 6-diamidino-2-phenylindole (DAPI, 1:400, Sigma, USA), nuclear staining was done for 15 minutes at room temperature. Ultimately, the scaffolds were washed three times with PBS and then examined by an invert fluorescent microscope (IX 71, Olympus, Japan).

Ethical considerations

This research was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1396.282). All protocols such as animal care, anesthesia, and euthanasia were performed in accordance with the guidelines of the moral committee of this university.

Statistical analysis

All data was expressed as the mean ± standard deviation and performed in triplicate and repeated three times with similar results. The data was analyzed using different techniques including one-way analysis of variance (ANOVA) followed by Tukey's test for each paired experiment. All analyses were done using GraphPad Prism Software (version 5.1, Graphpad Software Inc., La Jolla, CA, USA). Moreover, $P<0.05$ was considered statistically significant.

Results

Characterisation of decellularized muscle ECM

Macroscopic evaluation indicates discoloration of muscle tissue due to elimination of the muscle cells. Figure 2B shows that decellularized muscle scaffold was obtained, as a transparent sheet-like layer after completing all the steps of cell removal, washing and drying (for 6 days).

Images obtained from light microscope with H&E,

Masson's Trichrome staining, and SEM showed cell-free muscle tissue ECM. In addition, SEM indicated the porous space and intertwined fibers appearance of the decellularized muscle tissue. Different sections and views of the decellularized muscle ECM are shown in Figure 2B.

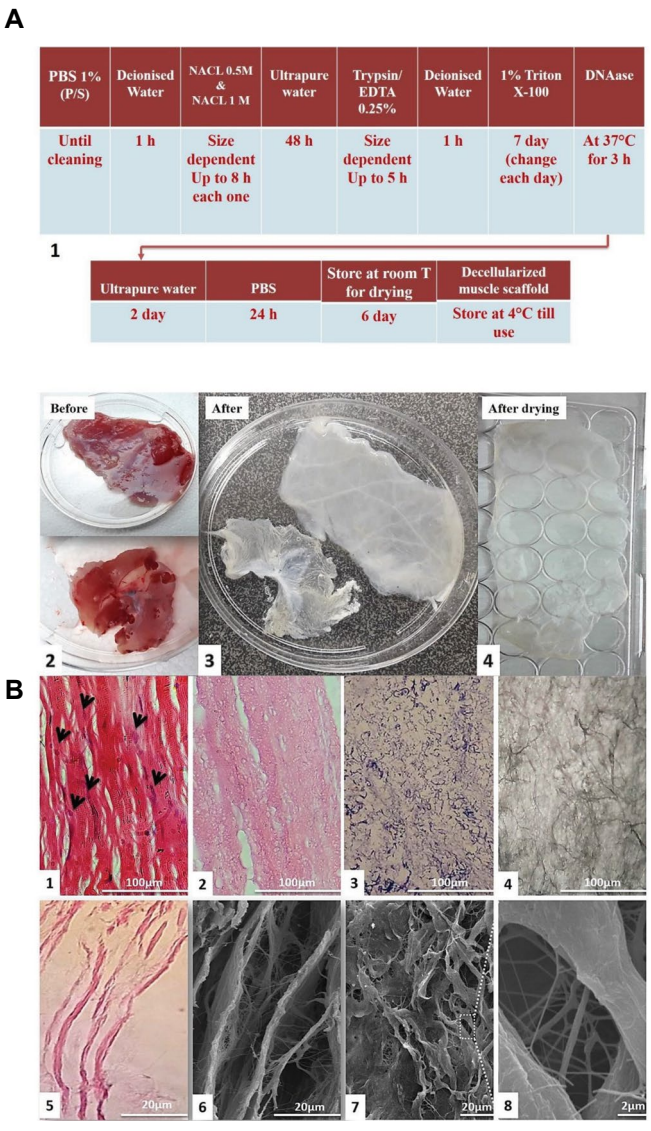
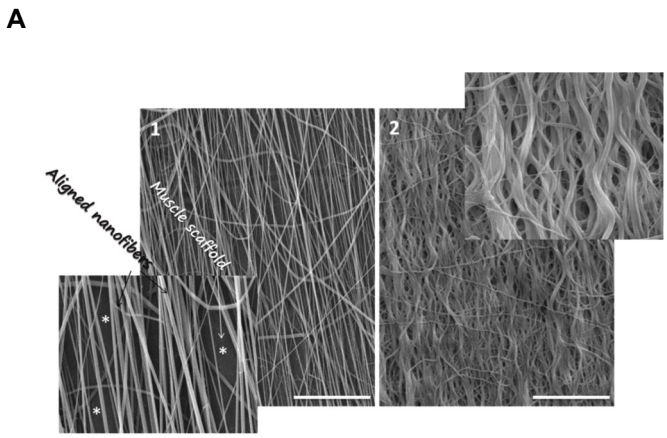


Fig.2: Macroscopic and microscopic assessment of the muscle tissue decellularization. **A.** The skeletal muscle tissue decellularization steps using Trypsin and Triton X-100 (as the important step), **A1.** Duration of each step depended on the size and thickness of the muscle tissue, **A2.** The diaphragm and the anterior abdominal wall muscles before decellularization, **A3.** After complete decellularization color clearness can be easily detected macroscopically, **A4.** The dried state of the decellularized muscle (ECM of the muscle tissue) prepared as a transparent layer. **B.** Light microscopic view and SEM of the normal and decellularized muscle tissue, **B1.** The normal abdominal wall anterior muscle of the rats in which the nuclei positions are shown with arrows (scale bar: 100 µm), **B2, B3.** The decellularized muscle tissue stained with H&E) Trichrome (scale bar: 100 µm), **B4.** Non-stained with light microscope (scale bar: 100 µm), **B5.** The longitudinal cross-section of the decellularized muscle tissue with light microscope (scale bar: 20 µm), **B6.** And with SEM (scale bar: 20 µm), **B7.** The surface view of the decellularized muscle tissue with SEM indicating the lack of any specific surface orientation (scale bar: 20 µm), and **B8.** A part of image B7 after magnification (scale bar: 2 µm). ECM; Extracellular matrix and SEM; Scanning electron microscope.

Viability of ADSCs/HUVECs Co-cultured on the scaffold engineered with PCL/gelatin nanofibers was evaluated through measurement of the cells' metabolic activity using the MTT test. The results indicate the relative improvement of ADSCs/HUVECs viability in comparison with the control group. Although the difference was insignificant, the similarity of culture conditions in 3D and 2D culture in growth, proliferation, and viability indicated the lack of toxicity of the decellularized muscle ECM. The results of mechanical tests showed a noticeable difference between the tensile strength of wet scaffolds compared to dry scaffolds. Also, ECM engineered with PCL/Gelatin were more resistant than ECM engineered without PCL/Gelatin nanofibers in dry and wet conditions, respectively (Fig.3).



		Force (N)	Extension (mm)	Stress (MPa)	Elongation (%)	Module (MPa)	Time (Min)
Decellular Muscle Dry	Peak	7.33	0.769	2.77	2.56	108.40	0:3.14
	Break	6.22	0.509	2.35	1.69	138.83	0:4.70
Decellular Muscle Wet		Force (N)	Extension (mm)	Stress (MPa)	Elongation (%)	Module (MPa)	Time (Min)
	Peak	4.76	6.741	2.16	21.08	10.26	0:39.00
	Break	4.58	6.483	2.08	20.27	10.28	0:40.59
Decellular Muscle & Nanofibers Dry		Force (N)	Extension (mm)	Stress (MPa)	Elongation (%)	Module (MPa)	Time (Min)
	Peak	9.55	1.027	2.09	3.54	59.14	0:4.68
	Break	7.95	0.76	1.74	2.64	65.94	0:6.24
Decellular Muscle & Nanofibers Wet		Force (N)	Extension (mm)	Stress (MPa)	Elongation (%)	Module (MPa)	Time (Min)
	Peak	3.68	8.56	0.80	25.96	3.11	0:53.04
	Break	3.62	8.53	0.75	25.22	4.23	0:54.22

Fig.3: Morphological and mechanical characterization of engineered muscle scaffold. **A.** Arrangement and bio-degradation behavior of the PCL/gelatin nanofibers; the images show SEM of the PCL/gelatin parallel nanofibers on the decellularized muscle scaffold; **A1.** The initial state (*) indicates the underneath muscle tissue covered by the parallel nanofibers (scale bar: 20 µm), **A2.** After culture in the incubator for 7 days to evaluate the rate of nanofibers biodegradation. The results demonstrated that the direction of the nanofibers did not change and were still in parallel, although their dimensions (swelling) and high adherence to each other show the proper bio-degradation trend of the PCL/gelatin nanofibers that can be considered a favorable feature for implantation (scale bar: 20 µm) and **B.** Stress-strain data of muscle ECM with and without PCL/Gelatin nanofibers in dry and wet status under tensile loading (scale bar 20 µm). ECM; Extracellular matrix, SEM; Scanning electron microscope, and PCL; Polycaprolactone.

PCL/Gelatin nanofiber arrangement

SEM images of electrospun PCL/gelatin nanofibers showed the parallel and unidirectional fibers arranged

on the muscle ECM before and after immersing in culture medium (Fig.3A). The nanofibers diameter was measured in the dry and wet states using Image J software indicating a mean diameter of 350 nm and 520 nm, respectively. As shown in Figure 3A2, although nanofibers were swelled and their thickness was increased, they were still in alignment, and favorable for myogenic differentiation.

Flow cytometry

After 4 passages, the expression of the isolated stem cell surface markers, including CD44, CD45, CD73, and CD90, was evaluated through flow cytometry; the results of which showed that CD44, CD73, and CD90 were expressed on the surface of these cells and almost a negligible number of the cells were positive for the hematopoietic cell marker CD45. All these results indicate the high purity of the isolated mesenchymal stem cells.

Co-culture and differentiation of ADSCs/HUVECs in 3D culture

In the 3D culture, ADSCs/HUVECs were cultured on the muscle ECM engineered with PCL/gelatin nanofibers in different culture media (proliferation or differentiation) for 1 week according to the experimental groups. Each group of endothelial cells (stained with C7000 cell marker) and mesenchymal cells (stained with tropomyosin antibody) in monoculture and co-culture states were evaluated. As shown in Figure 4, the growth and cell proliferation of HUVECs in the HS group was much lower than the VEGF group. The results from ADSCs groups indicated myogenic differentiation of ADSCs to myoblast-like cells and the expression of tropomyosin on the engineered scaffold in the monoculture and co-culture. In addition, it was shown that the differentiated cells in the Co-culture/ HS/VEGF group were arranged close to the HUVECs on the engineered structure. Although no geometrical vascular structure was observed, their proliferation and distribution in the VEGF-differentiated group was well observable. VEGF in the Co-culture groups also increased the myogenic induction and hence lead endothelial cells to be in close proximity with differentiated myoblast-like cells (Fig.4).

Scanning electron microscopy

Seven days after differentiation, the position and arrangement of the ADSCs and HUVECs on the engineered scaffold with PCL/gelatin parallel nanofibers were examined through SEM. The results revealed that the ADSCs/HUVECs co-culture had a higher growth and proliferation in the VEGF-treated group. Based on the cells' dimensions, it can be concluded that the higher number of endothelial cells (signified with *) was the reason for the higher density in the VEGF group, which indicates the capacity of this cell model for providing the angiogenesis process in the engineered scaffold. These cells were located on the PCL/gelatin parallel nanofibers and attached to each other and to the scaffold underneath. In the absence of VEGF, proliferation of the cells was low which was consistent with the findings obtained from immunofluorescence technique (Fig.5).

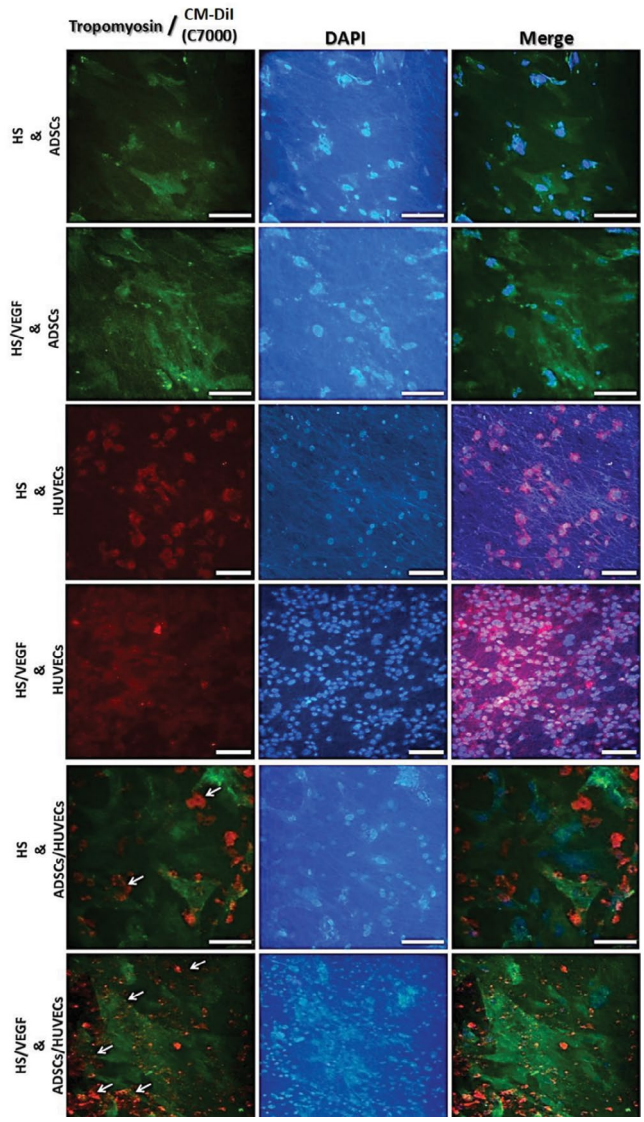


Fig.4: Immunofluorescence and Morphological characterization of ADSCs, HUVECs and HUVECs and ADSCs co-culture in experimental groups (HS and HS/VEGF). The expression of tropomyosin in HS and HS/VEGF groups was indicated in immunofluorescence. As shown, ADSCs proliferation was increased in HS/VEGF group and were arranged in proximity to parallel nanofibers. As shown in figures, the proliferation and distribution of HUVECs in the HS/VEGF group was increased in comparison to the HS group which indicates the predictable effect of VEGF on HUVECs. Also the orientation of the PCL/Gelatin nanofibers can be detected. In HUVECs and ADSCs co-culture, Tropomyosin-specific fluorescent staining for differentiated mesenchymal cells and endothelial cells-specific C7000 staining are shown. The density of differentiated myoblasts like cells, in particular endothelial cells, was higher in the VEGF-differentiated group compared with the HS-differentiated group after the 7 days differentiation period. The endothelial cells are signified with red. The arrows indicate the level of distribution and proliferation of HUVECs in the groups. As is obvious, like 2D culture, growth and proliferation of the endothelial cells along with myogenesis differentiation of the ADSCs on the VEGF-differentiated group is well observed (scale: 200 µm).PCL; Polycaprolactone, ADSCs; Adipose-derived stem cells, HUVECs; Human umbilical vein endothelial cells, HS; Horse serum, and VEGF; Vascular endothelial growth factor.

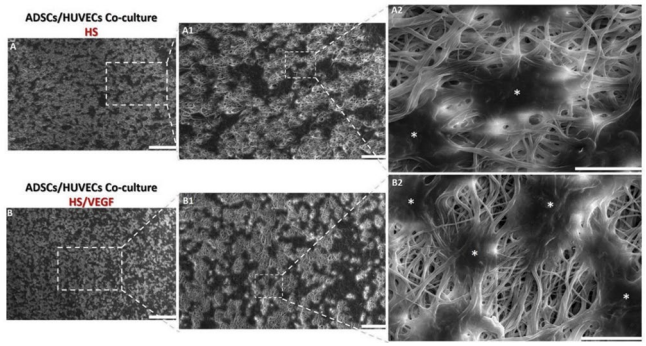


Fig.5: SEM of the ADSCs/HUVECs co-cultured on the muscle ECM engineered with PCL/gelatin parallel nanofibers after a-week of differentiation in 3 different magnifications. The cell density is higher in image B (VEGF group) compared to Image A (HS group). Based on the cell size, (*) indicates endothelial cells. Proliferation and distribution of these cells in the VEGF-treated group was much higher than the HS-treated group (scale bar of Fig.A, B: 200 µm, Fig.A1, B2: 20 µm, Fig.A2, B2: 10 µm). PCL; Polycaprolactone, ADSCs; Adipose-derived stem cells, HUVECs; Human umbilical vein endothelial cells, ECM; Extracellular matrix, HS; Horse serum, SEM; Scanning electron microscope, and VEGF; Vascular endothelial growth factor.

Results of real-time RT-PCR in the 3D culture

The quantitative data obtained from real-time RT-PCR in the 3D culture on nanofibers proved that the expression of *MyHC2* was significantly higher in the VEGF and HS groups as compared with the control group (P<0.001). In addition, there was a significant difference between the VEGF and HS groups in terms of expression of this gene (P<0.05). As an important point of these findings, expression of *MyHC2* in the presence of VEGF was increased in the ADSCs/HUVECs co-culture in comparison with the ADSCs monoculture (P<0.05). However, there was no significant difference between the HS-treated group in co-culture and ADSCs monoculture groups in *MyHC2* expression. These results indicated that VEGF along with HUVECs can increase the expression of *MyHC2* in the mesenchymal differentiated group as compared with the other groups (Fig.6). It should be mentioned that although HUVECs monoculture (without VEGF) were unable to significantly improve *MyHC2* gene expression, the co-culture along with VEGF resulted in a significant difference in comparison with ADSCs monoculture (P<0.05). This finding indicates the effective role of ADSCs/HUVECs co-culture along with the use of VEGF in increased expression of the myogenic differentiation genes. The results of comparison between 2D and 3D cultures in different groups showed that the *MyHC2* gene had a significantly higher expression in the 3D culture in comparison with the 2D culture, so that its expression was significantly increased in the HS/3D group compared to the HS/2D group (P<0.05) and in the HS/VEGF/3D group compared to the HS/VEGF/2D and other groups (P<0.05, Fig.6).

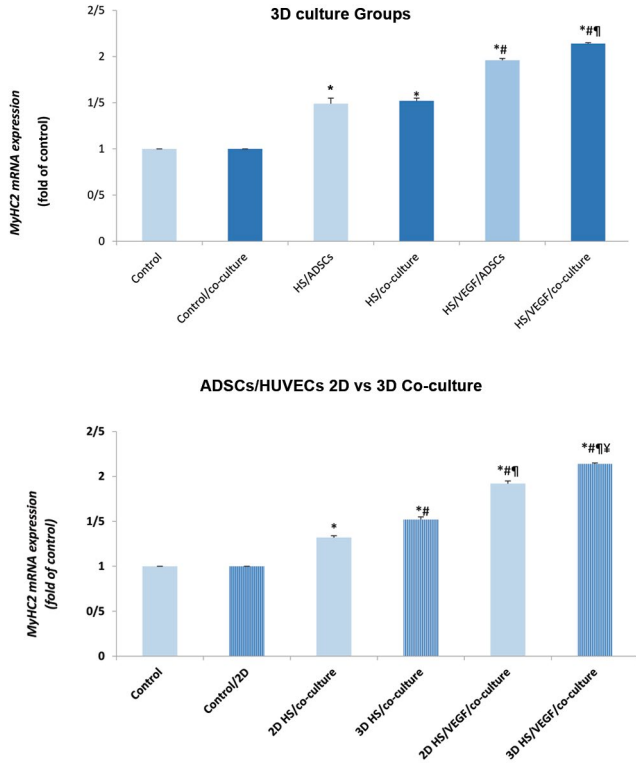


Fig.6: Gene expression in experimental groups. Expression of *MyHC2* mRNA in different experimental groups which was 1.49 ± 0.06 in the HS/ADSCs group, 1.52 ± 0.03 in the HS/Co-culture group, 1.96 ± 0.02 in the HS/VEGF/ADSCs group, and 2.14 ± 0.01 in the HS/VEGF/co-culture group, *; P<0.001, #¶; P<0.05; *; Indicate the comparison between the control groups. #; Indicate the comparison between the HS groups, and ¶; Indicate the comparison between the HS/VEGF groups. Expression of *MyHC2* mRNA in 2D and 3D cultures which was 1.32 ± 0.02 in the HS/2D group, 1.52 ± 0.03 in the HS/3D group, 1.92 ± 0.03 in the HS/VEGF/2D group, and 2.14 ± 0.01 in the HS/VEGF/3D group. *; P<0.001, #&¶&¥; P<0.05, *; Indicate the comparison between the control groups, #; Indicate the comparison between the HS/2D groups, ¶; Indicate the comparison between the HS/3D groups, ¥; Indicate the comparison between the HS/VEGF/2D groups, ADSCs; Adipose-derived stem cells, HUVECs; Human umbilical vein endothelial cells, HS; Horse serum, and VEGF; Vascular endothelial growth factor.

Discussion

Skeletal muscle tissue engineering is a complex process and achievement of a favorable and contractive tissue requires some prerequisites such as formation of myofibrils, development of vascular structures, innervation, and improvement and organization of the ECM (18).

In this study, the effects of endothelial cells (HUVECs), VEGF, and engineered muscle ECM with PCL/gelatin nanofibers was investigated on myogenic differentiation of the ADSCs. The use of such a scaffold along with ADSCs/HUVEC co-culture in evaluation of myogenic differentiation and their mutual roles to improve angiogenesis have not been investigated so far. The muscle scaffold or ECM was used given numerous studies performed in this regard. ECM is usually obtained through decellularization of the tissues and organs of mammals. For example, ECM of intestine, dermis, bladder, pericardium, heart valves, amniotic membrane, muscle, and fascia are approved by FDA to be used for soft tissue regeneration (19). Li et al. (20) reported that the

use of dehydrated amniotic membrane in damaged biceps femoris of rats caused migration of a higher number of myoblasts to the affected area. In another study, Merritt et al. (21) evaluated the role of muscle-derived ECM in the improvement of the gastrocnemius muscle function in Lewis mice and showed a function improvement rate of 85% after a period of 42 days, indicating the effective therapeutic role of the ECM and BMSCs combination in regeneration of skeletal muscle. In this regard, Badylak et al. (22) used a medical regeneration approach on 13 patients with muscle defects and showed that the contractive force increased by 37.3% and muscle motility improved by 27.1% after using muscle ECM in the damaged area and physiotherapy exercises. According to previous studies and the confirmed effective role of decellularized scaffold, abdominal muscle tissue ECM was used in this study, so that after decellularization of the tissue, a clear cell-free structure was obtained and used for *in vitro* experiments. In this study, a transparent muscle scaffold was obtained that had a significant role in myogenic differentiation (as increased expression of the *MyHC2* gene) in ADSCs compared with 2D culture. The muscle ECM is an ideal option in muscle tissue engineering due to its specific properties.

Wang et al. (17) compared the decellularized muscle and fascia tissues in their study and showed that both tissues not only maintained their 3D optimal structure, mechanical properties, and biologic compounds, but also were non-toxic and well retained their ability to induce the integrity of human adipose stem cells (hASCs). In addition, they reported that the muscle scaffold has a significant role in induction of angiogenesis and the pro-myogenic properties in comparison with the fascia scaffold, which can be considered as a favorable scaffold for engineering muscle and fascia tissues.

It should be noted that in addition to the scaffold material and ECM properties, the surface topography is an important parameter for muscle tissue engineering. Regular and unidirectional scaffolds are more potent to form myotubes and regular arrangement of myofibers than scaffolds with multidirectional fibers on their surface. Chen et al. (23) used a new type of 3D porous collagen scaffold with special microgrooves in their research, which formed a structure similar to the muscle basement membrane for skeletal muscle tissue engineering. In our study, the topography of the muscle scaffold due to lack of no specific direction was engineered to create a favorable and parallel shape for myogenic differentiation using PCL/gelatin parallel nanofibers electrospinning. PCL/gelatin is an ideal compound for tissue engineered, because PCL increases the structural strength and gelatin can improve cell attachment. Kim et al. (13) showed that the expression rate of myosin heavy chain and troponin T in C2C12 cells and their proliferation rate increased significantly when the PCL/gelatin scaffold was used as compared with the PCL scaffold. Likewise, Yang et al. (12) showed that human ADSCs were arranged longitudinally in parallel with the electrospun PCL/gelatin nanofibers and were able to acquire tendon cell phenotypes under the influence of TGF-β3. Our research indicated a better arrangement of ADSCs unidirectional with the parallel nanofibers and expression of differentiation factors, in particular the expression of *MyHC2* gene in the HS and HS/VEGF differentiation groups both in the monoculture and co-culture as compared with the 2D culture (lacking the parallel nanofibers). It seems that using VEGF could be helpful in the muscular-vascular differentiation process. VEGF is a key regulator of angiogenesis, but its effect on restoration of muscular force and musculoskeletal damages regeneration has not yet been properly identified. Recent studies have shown that VEGF may affect a lot of different cells including neurons, hepatocytes, osteoblasts, hematocytes, and myoblasts (24). In addition, it was proven that in vitro administration of VEGF can stimulate migration of myoblasts, improvement of cell life critical conditions, protection of myogenic cells against apoptosis, and progression of myoblast growth (15). Chen et al. (25) and Song et al. (26) reported in two independent studies that VEGF is the main factor in differentiation of embryonic stem cells (ESCs) and ADSCS to cardiomyocyte, respectively.

The results of this study showed that VEGF significantly increased the myogenic differentiation of ADSCs in monoculture or co-culture as compared with other differentiation or control groups.

Shvartsman et al. (27) implanted VEGF- and IGF1-including alginate gels to the skeletal muscle ischemic damage area and showed that they were able to increase the regeneration speed and progression of angiogenesis process and helped viability of myoblasts. Kim et al. (28) showed that VEGF associated with ADSCs can be used as an angiogenesis strategy in tissue engineering specially the complex muscle tissue.

The results of this study are consistent with those of other studies in this field, so that based on our study, VEGF induced myogenic differentiation in ADSCs and endothelial tube formation in the co-culture groups under the influence of HS/VEGF. The endothelial tube was observed as a regular pentagon shape initiating the angiogenesis process in the co-culture groups in the 2D culture. This information followed a scientific fact, that angiogenesis is the prerequisite of favorable myogenesis so that myogenesis seems to be increased in groups where VEGF was used due to improved vascular structure (although primitive). As an important point, angiogenesis and myogenesis were induced by VEGF. This was also mentioned in previous studies, so that increased angiogenesis by VEGF improves the muscle function in ischemic tissues (37). The findings of this study are similar with previous studies regarding the relationship between angiogenesis and more effective regeneration of muscle tissue (29).

In this study, endothelial cells were co-cultured with ADSCs with two objectives: first, to evaluate their roles

in myogenesis differentiation of ADSCs and second, to provide conditions for angiogenesis and vascular organizations by HUVECs using VEGF (or paracrine effects of ADSCs) in the engineered structure. Co-culture of ADSCs and HUVECs was performed at a ratio of 1:1. This ratio was chosen according to previous studies on mesenchymal cells and HUVECs (30) as well as myoblasts and HUVECs (31). Accordingly, the cell-to-cell interaction and the paracrine effect of endothelial cells on differentiation, morphology, and direction of ADSCs and vice versa was investigated. This subject was raised based on the idea that cell-to-cell interactions between ADSCs and HUVECs may improve myogenesis differentiation. In this regard, Lin and Lilly (32) reported that co-culture of endothelial cells and bone marrow-derived stem cells (BMSCs) can guide BMSCs toward smooth muscle cells due to their role in advancement of contractive phenotype, decrease in proliferation, and increase in synthesis and release of collagen fibers. Accordingly, Gholobova et al. (33) indicated that co-culture of endothelial cells and skeletal muscle precursor cells on engineered ECM can improve the parallel and dense arrangement of muscle fibers which led to expansion of the endothelial vascular network (in particular in a high cell density).

Similarly, Chen et al. (31) reported that myoblasts and HUVECs co-culture could cause a significant increase in the myosin heavy chain expression rate and parallel formation of myoblasts along with regular arrangement of vascular endothelial tubular cells in the porous scaffold.

Koffler et al. (34) used a triple culture system consisting of myoblasts, HUVECs, and fibroblasts and observed the formation of muscle tissue and vascular network, organization, and integrity of the host tissue 3 weeks after implantation. Other studies have shown that co-culture of myoblasts with endothelial cells in a sandwich-like structure improved the formation of vascular structures (35). Criswell et al. (18) investigated the co-culture of HUVECs and precursors of 10T1/2 myoblasts on an acellular bladder scaffold *in vitro* and *in vivo* and showed that the rate of angiogenesis, innervations, and myogenesis significantly increased in comparison with scaffolds cultured with only myoblasts.

The present study proved that although VEGF-free HUVECs were not able to significantly improve the expression of *MyHC2* gene, their simultaneous co-culture with the use of VEGF led to significant difference in comparison with the ADSCs monoculture. This finding indicates the effective role of ADSCs and HUVECs co-culture on the expression of differentiation genes especially when VEGF was used. It should be noted that there are scarce information about co-culture of ADSCs/HUVECs in the myogenic differentiation process, and most studies focused on the muscle precursor cells. As another important point, this study investigated the role of HUVECs on myogenic differentiation and ADSCs direction as well as the mutual connection of these cells aiming to develop an engineered vascular-muscle cellular model, a process that has not been studied yet. This study intended to design a favorable cell model using a cell-free muscle scaffold aiming to develop a vascular-muscle structure and take a step toward solving the extensive challenges of muscle tissue.

Angiogenesis and formation of vascular network in engineered tissues has been always an important challenge in recent years. Therefore, this study evaluated the formation of vascular structures of endothelial tube formation in 2D Co-culture. We assumed that the interaction between ADSCs and HUVECs may result in proliferation and improvement of cell arrangement as well as angiogenesis by the endothelial cells, and all these processes may improve myogenic differentiation. It is believed that endothelial cells are induced to initiate the angiogenesis process, as endothelial tube formation, during regeneration of vascular structures *in vitro*.

Recent studied have reported that the co-culture of ADSCs/ECs can induce the formation of vascular sprouts and significantly increase the number of connections and vascular tubules. According to studies, induction of these vascular structures may arise from factors produced by ADSCs and released in the culture medium. Using ELISA technique, Holnthoner et al. (36) showed that an increased number of ADSCs in the co-culture results in an increase in the VEGF concentration in the culture medium. It has been proven that ADSCs release a large amount of VEGF into their culture medium (25). Numerous studies have reported that simultaneous induction of muscle precursor cells and formation of vascular tubules can be stimulated due to the paracrine effects of VEGF as well as IGF-1, HGF, bFGF, and PDGF-BB (37). Contrary to our findings, Kook et al. (38) did not observe any capillary network after Co-culture of ADSCs/HUVECs in cell plates. They reported a slight increase in proliferation, expression of adhesion proteins, and vascular branches originating from HUVECs in the VEGF-treated co-culture group. In contrast to the results of Kook et al. (38) our findings confirmed that ADSCs can induce the formation of endothelial tube formation in the VEGF-treated co-culture group (in 2D culture) significantly more than other groups (lacking VEGF). This finding was similar to that of Park and Gerecht (39), probably due to direct cellular interaction and more importantly due to the effects of ADSCs paracrine secretions such as VEGF, angiopoietin 1 and 2, and interleukin 6 which result in cell proliferation and endothelial tube formation in HUVECs. Finally, after accurately reviewing all aspects in this study, it can be concluded that this research confirms other studies in this regard: on the one hand, its provides a favorable cellular model in the framework of an engineered ECM muscle scaffold and on the other hand, it was able to take a step towards designing an effective engineered vascular-muscle structure.

Conclusion

It can be generally concluded that muscle ECM engineered scaffolds can be used as a desirable strategy in muscle tissue engineering. Also, ADSCs / HUVECs

cell interaction and dual effects of VEGF can result in the myogenic differentiation of ADSCs in close proximity to endothelial cells and vascular network. Co-culture of ADSCs/HUVECs can be an ideal option for achieving a favorable cellular model for use in the engineered vascular-muscular tissues. Redesigning such engineered structures can be used in challenging muscle injuries such as VML, where muscle and vascular structures regeneration are required.

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Authors' Contributions

A.H.M., V.B., M.O.; Participated in study design, data collection and evaluation, drafting and statistical analysis. A.H.M., V.B., M.R.; Contributed to all experimental work such as muscle decellularization, cell culture, immunofluorescence staining, and interpretation of data. A.H.M., M.R.; Conducted molecular experiments and RT-qPCR analysis. V.B., M.O.; Were responsible for overall supervision. A.H.M.; Drafted the manuscript, which was revised by V.B., M.O. All authors read and approved the final manuscript.

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