

Enriched Satellite Cells with Pre-plate Technique Differentiate Strongly on Electrospun Polyacrylonitril Membrane

Simzar Hosseinzadeh ^{a,b*}, Masoud Soleimani ^c

^a School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; ^b Stem Cell Biology Department, Stem Cell Technology Research Center, Tehran, Iran; ^c Hematology Department, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran

*Corresponding author: Simzar Hosseinzadeh, Department of Tissue Engineering, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Daneshjou Boulevard, Evin, Tehran, Iran, P.O.19839. **E-mail:** s.hosseinzadeh@sbmu.ac.ir; **Tel:** +982122439847

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Introduction: Satellite cells known as the main regenerating cell types in skeletal muscle which can be isolated using pre-plate technique due to weak or slow adhesive interactions with satellite cells. Although, there are some issues about digestion of muscle tissue and isolation of satellite cells, which highlight need for an efficient procedure. Also, the employment of a nanofibrous surface can facilitate the attachment of satellite cells to reach matured muscle tissue. On the other hand, polyacrylonitrile (PAN) has been reported as a biocompatible polymer that can be electrospun into a nonwoven membrane. **Materials and Methods:** Herein, a modified digestion and pre-plate protocol was established for the enrichment of satellite cells. Also, a PAN electrospun scaffold was used to provide a higher surface area for cell attachment compared to tissue culture polystyrene (TCPS). However, the surface of prepared scaffold was modified with plasma treatment to progress cell adhesion. **Results:** The corresponding scaffold was examined with scanning electron microscopy (SEM) and tensile examination. The enriched cells, which exhibited a close gene expression pattern with satellite cells, seeded on this electrospun PAN membrane. The cultured satellite cells showed a good tendency to surface of PAN scaffold and also a higher rate of cell proliferation. Subsequently, the cells were induced to more expression of specific muscle genes compared to TCPS group. **Conclusion:** As a whole, satellite cells could mature to multinuclear cells using PAN scaffold as a function of efficient mechanical property and also higher surface area.

Keywords: Polyacrylonitrile; Electrospinning; Skeletal muscle; Satellite cells; Pre-plate technique; Stem cells

Introduction

The skeletal muscle tissue may be affected by myopathies some severe diseases such as Duchenne's Muscular Dystrophy (DMD), aging or exposure to myotoxic agents, sharp or blunt trauma, ischemia, and hot or cold temperatures (1, 2). Satellite cells are progenitor populations of quiescent mononucleated stem cells in skeletal muscle, which are located between the basal lamina and plasmalemma of muscle fibers (3-5). In response to muscle injury, satellite cells become activated and proliferate and finally fuse together to form new myofibers (6-8). Also, satellite cells can differentiate into other lineages, such as osteoblastic, adipocytic (9), and neural cells (10). In before studies, satellite cells have been introduced by the expression of a self-renewal signal, Pax7 and regulators of myogenic commitment, MyoD. When satellite cells differentiate, the expression of Pax7 reduces while MyoD is up-regulated with the early stages of regenerative myogenesis (11). Similarly, the expression of M-cadherin is increased over myogenesis following injury (7). Further on, satellite cells express

CD34 as a common marker of endothelial cells (12) which is down regulated during muscle regeneration (13).

For muscle tissue engineering, an appropriate scaffold is required to improve the cell proliferation and maturation (14, 15). The corresponding scaffold should provide good mechanical properties as well as a higher ratio of surface area to volume. Electrospinning technique can produce nanofibers to reach a higher level of mimicry of extracellular matrix (ECM). The electrospun nanofibrous membrane is one of the best candidates for versatile applications including tissue engineering, filtration, reinforcement in composite materials (16), fuel cells, hydrogen storage, sensors, and batteries (17-19). Polyacrylonitrile (PAN) fibers have been defined in many high technology applications such as; water absorbents, ion exchange materials, and precursors for the production of carbon fibers. In the present study, isolated satellite cells using a pre-plate technique were cultured on the PAN electrospun scaffold. The results related to molecular and cellular assays, confirmed the higher cell viability and also muscle maturation of satellite cells.

Materials and Methods

Digestion of skeletal muscle

Approximately 2-3 naval medical research institute (NMRI) mice were sacrificed at 10 days after birth and skeletal muscles were obtained in the following from fore and hind feet. Non-muscle tissues inclusive adipose and dermal tissues were gently removed, then incubated with Trypsin (0.25% Trypsin/1 mM EDTA) (Gibco) for 4 minutes for complete digestion of tissue samples, an enzymatic dissociating solution including collagenase type1 (Gibco), collagenase type 4 (Gibco) 0.1% and dispase 1.75 unit/mg (Gibco) were recruited for an overnight at 4°C. Then, the sample was stored for 3.5 hours in an incubator at 37°C and a 70µm mesh (BD Falcon) was utilized to filter. The obtained suspension was centrifuged at 1250 RPM for 5 minutes and divided equally between two 25-cm² flasks of cells after removal of supernatant. Finally, the assessment of pre-plate performance was done to check the intrinsic of isolated cells and compared with a subcultured group (non-pre-plated cells).

Isolation of satellite cells by pre-plating method

The single-cell suspension was cultured by adding medium consisting of DMEM-high/F12 (Gibco) with 10% FBS. The cells were incubated at 37°C and after 1.5 hours, the first pre-plate protocol was performed by adding of PBS-EDTA (Sigma). After centrifugation of detached cells at 1250 RPM for 5 minutes, the cells were resuspended with medium consisting of DMEM-high/F12 with 10% FBS and plated the cells into a gelatin (Sigma)-coated flask. The procedure was repeated every day and the enriched cells from 6th pre-plating were used for detection by real-time PCR, flow-cytometry, and immunostaining. For satellite cells delivery on prepared scaffold, the cells were counted and cultured with confluent of 12×10³ cells per well.

Molecular technique for characterization of skeletal muscle enriched cells

Real-time PCR was done for β-actin as internal control along with Pax7, MyoD, α-actin, MyH, M-cadherin, and CD34 as skeletal muscle markers on 1th, 6th, and 12th days. The following primer sequences are listed in Table 1. For extraction of RNA and cDNA synthesis, TRIzol reagent (Sigma) was used to isolate total RNA and according to the manufacturer's instructions (Fermentas), cDNA was synthesized with M-MuLV reverse transcriptase (RT) and Random Hexamer primers. The PCR reactions 94°C for 3 minutes as annealing temperature, 35 cycles as 94°C for 30 seconds, 62°C for 45 seconds, 72°C for 45 seconds and the extension time was 7-10 minutes with 72°C were conducted with 0.5 µl of the cDNA product. Real-time PCR reactions performed using Maxima™ SYBR Green/ROX Real-

Time PCR Master Mix (Fermentas) and Rotor-gene Q software (Corbett) for data analysis of threshold cycle average. Gene expression levels were calculated based on the ΔΔCt method.

In Vitro differentiation of isolated satellite cells

For osteogenic differentiation, the cultured cells from 6th pre-plating were treated with bone-morphogenesis proteins (Dexamethasone 10⁻⁷ M, Beta-Glycerol-Phosphate 10 mM and Ascorbic Acid bi-Phosphate 50 µg/ml). Also, for adipogenic differentiation, the conditioned medium with adipogenic-inducing agents including IBMX 0.5 mM, Dexamethasone 10⁻⁷ M, Insulin 66 nM, and Indomethacine 0.2 mM were used for adipogenic differentiation. Neural differentiation was induced using isobutyl methyl xanthin: IBMX 0.5 mM, Forskollin 10 µM and Retinoic Acid 10⁻⁶ M. The differentiated cells were presented by immuno-cytochemistry for specific markers of neural, microtubule-associated protein-2 (Leinco Technologies), and βtubulin 3 (abcam). For this assay, the cells were fixed with 4% paraformaldehyde (Sigma). Triton X100 (Sigma) 1% was added and the samples were kept at room temperature. For blocking step, we used 5% goat serum (Merck) for 45 minutes at room temperature. Then without washing, first antibodies were added (diluted in BSA (Merck)/PBS 0.2%), 4°C for an overnight. Tween 20 (Merck) 0.1% was added to wash the samples and followed by adding second antibody (diluted in BSA/PBS 0.2%), 3 hours at room temperature. The cells also were stained with DAPI (Sigma), and analyzed with an invert fluorescent microscope (Nikon TE-2000).

Scaffold preparation with electrospinning

For fabrication of PAN electrospun mat, 100 g of PAN (Aldrich with the average molecular weight of ca 100,000 Da) was dissolved in 1000 ml of DMF and stirred at 700 RPM in 25°C for 4 hours. The obtained solution was transferred to syringe with gage number of 22 and run with electrospinning device. The parameters including applied voltage, distance between needle and collector, injection rate (debi) and collector rotating rate were optimized. The obtained scaffold should have enough thickness to measure mechanical property and also cell culture process. The surface of ultimate scaffold was changed using plasma treatment with plasma generator with frequency of 44 GHz (Diener Electronics, Germany). The nanofibers were exposed to pure oxygen to provide oxidized groups at 0.4 mbar pressures and the glow-discharge was ignited for 5 minutes. The treated nanofibers were divided into 2 groups in the following: The non-plasma treated as control group and treated nanofibers as experimental type. The sheets were cut into 1.5cm diameter punches and immersed in ethanol (Merck, 70%) for 2 hours to sterilize the scaffold samples.



Characterization techniques of prepared nanofibrous PAN scaffold

SEM characterization method was carried out for fabricated scaffold types to show uniformity of resultant nanofibers (SEM, Philips XL30, Netherlands) after gold sputtering. Mechanical properties of corresponding scaffold were studied with Tensile machine (SANTAM (Iran, SPM20)). The mechanical assay was performed at a 50 mm/min crosshead speed. A digital micrometer was utilized to measure the thickness of electrospun samples, they were cut in a rectangular shape of 20 mm wide with 80 mm length and was used. Approximately, 0.5 kN was applied to the scaffold samples and the stress-strain curve was obtained in the following. The tensile strength, strain and young modulus (E) was calculated based on below formula:

Peak stress (MPs)=F/A

When F is the maximum load force (newton) and A (squared meter) is the cross-sectional area of samples.

Strain (%)=(L-L₀)/L₀×100

L₀ is the initial length of scaffold sample and L shows the elongated length before break point. The Young modulus was determined after measurement of slope of the stress-strain curve (20).

Cellular characterization of PAN scaffold after satellite cell culture

MTT assay for investigation of scaffold biocompatibility

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was utilized to expose cell viability on 1, 3, 7, and 15th days of cell culture. For this assay, cell-cultured scaffold inclusive plasma treated and non-plasma treated types as well as TCPS were rinsed by PBS and incubated with MTT dissolved in medium of DMEM without FBS at 37°C for 3.5 hours. The MTT solution was aspirated in the following and DMSO was added to dissolve formazan crystals. The absorbance at 570 nm was obtained using Microplate Reader.

SEM examination of satellite cells at 15th of culture on fabricated scaffold

The PAN scaffold which was seeded with satellite cells, cultured for 15 days and then, for micrographical assessment was submerged for 45 min. in a fixative solution containing 2.5% glutaraldehyde in PBS. Following the fixing, the samples incubated for 10 min. in 50% ethanol, 10 min. in 75% ethanol, 10 min. in 90% ethanol, and then 10 min. in 100% ethanol. The ethanol saturated samples were dried and the samples were coated in vacuum condition with gold (30sec, ~thickness 5nm). SEM morphological studies were used at an accelerating voltage of 5kV.

Statistical section

Statistical analysis was done by using SPSS software version 13 (SPSS Inc., Chicago, IL, USA). Mann-Whitney was used to assess differences between data means of experimental groups and control group. Each molecular and cellular assays were repeated at least for 3 times and the P-value of less than or equal to 0.05 was considered statistically significant. The data were presented as mean ± standard error.

Results

Morphological study of isolated satellite cells

After doing enzymatic tissue digestion, as described above, the cells are very highly dissociated (Figure 1a). By performing of second pre-plate, the remaining cells had the closely-packed appearance similar to epithelial cells (Figure 1 b-c). The other cell lineages like neural and adipose cells were removed after third pre-plate. The eventual cells of 4th and 5th pre-plates are very putative differentiating cells to large and multi-nuclear cells after 4-5 days (the specific morphology of the adult skeletal muscle cells) (Figure 1 d-e). By further enrichment, after 6th pre-plate, the cells had high potency to proliferate and owing to this, the growth factors like EGF (20 ng/ml) and bFGF (10 ng/ml) (10) had no significant impression on the proliferative potency of these cells and their differentiation was occurred in 12-15th day (Figure 1 f-g). Although, by applying 2% horse serum (21) to culture media, the committed muscle cells increased and cell fusion was observed after 3-4 days (Figure 1 h). On the other hand, the subculture group had high proliferative potency, but by much less propensity in giving rise to the adult muscle cells (Figure 1 i-j).

Gene expression profile of isolated satellite cells

In this study, the efficiency of pre-plate technique for isolation of satellite cells was evaluated by real-time PCR (Figure 2). The corresponding analysis was performed to compare between gene expression of pre-plated cells as test group and subculture cells (non-pre-plated cells) as a control group on 1th, 6th, and 12th day after digestion of skeletal muscle tissue. The gene expression was normalized with β-actin (the housekeeping gene in skeletal muscle cells). The comparison between separated groups (test and control) in figure 2 shows that among the targeted genes, at 6th day (the relative expression of 6th day to 1th day), Pax7 had the significant high expression level in pre-plated group (p-value<0.05). Besides, except CD34 and M-cadherin, other skeletal muscle markers, MyoD, MyH and α-sarcomeric actin were significantly upregulated in preplated



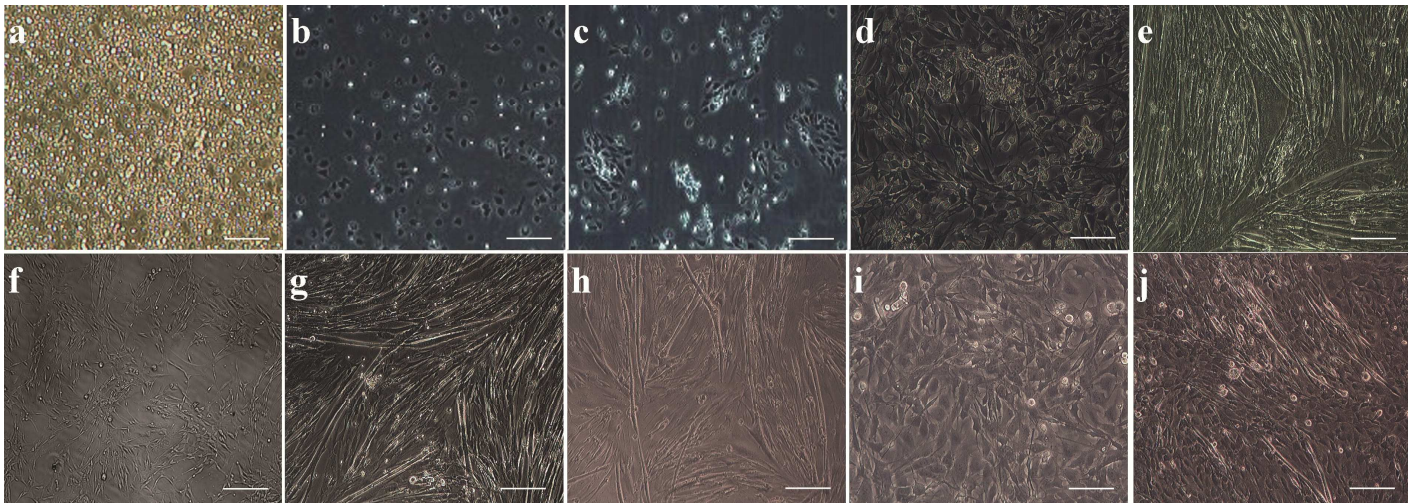


Figure 1. (A) The all cells of Skeletal Muscle after digestion period; (B) The remaining cells after first pre-plating; (C) The closely-packed appearance similar to cuboidal cells after second pre-plating; (D) The resulted cells from 5th pre-plating; (E) The spontaneous muscle differentiation of fifth pre-plating cells;(F) The resulted cells from 6th pre-plating; (G)The spontaneous muscle differentiation of 6th pre-plating cells; (H) The muscle differentiation of pre-plating 6 cells with 2% of Horse-Serum; (I) The subcultured cells as Control group; (J)The spontaneous differentiation of subcultured cells. Scale bar is 100 μm

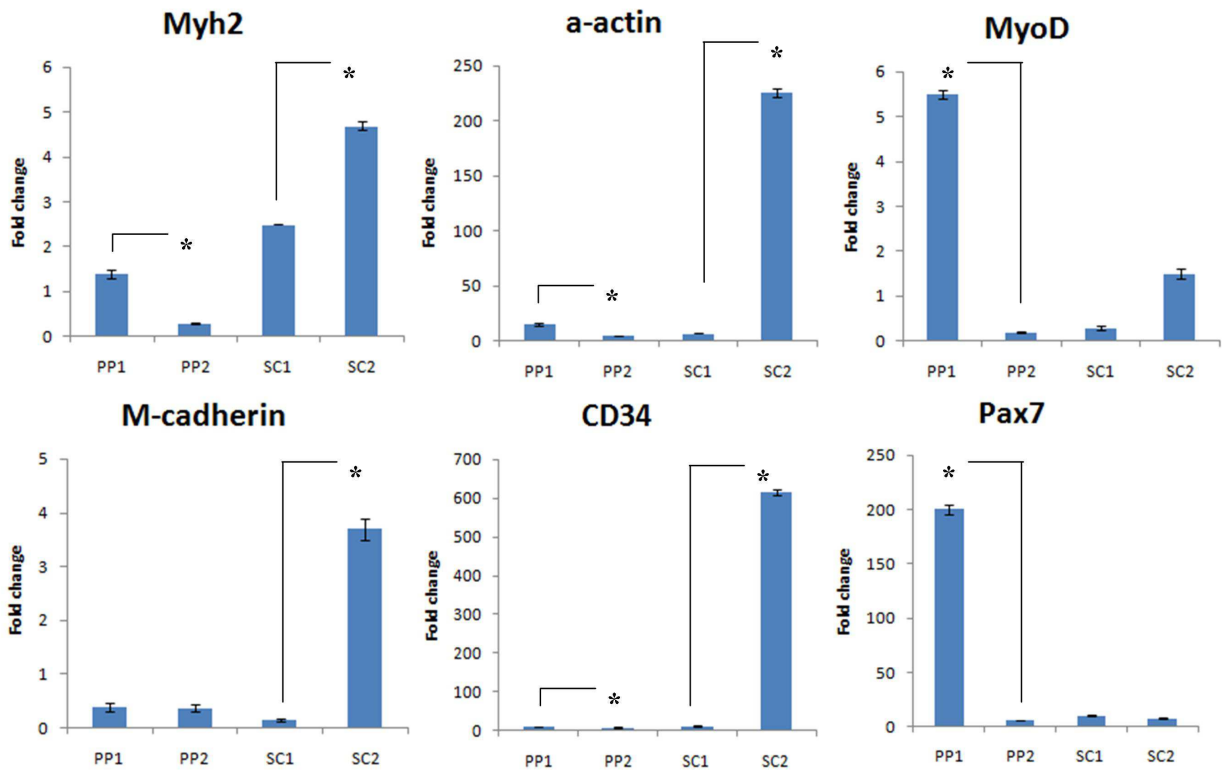


Figure 2. The Real-Time PCR shows relative expression of Markers for satellite cells (pre-plated and subcultured cells). Abbreviations: PP1;pre-

plated cells at 1th day; PP2;pre-plated cells at 12th day; SC1;subcultured cells at 1th day; SC2;subcultured cells at 12th day

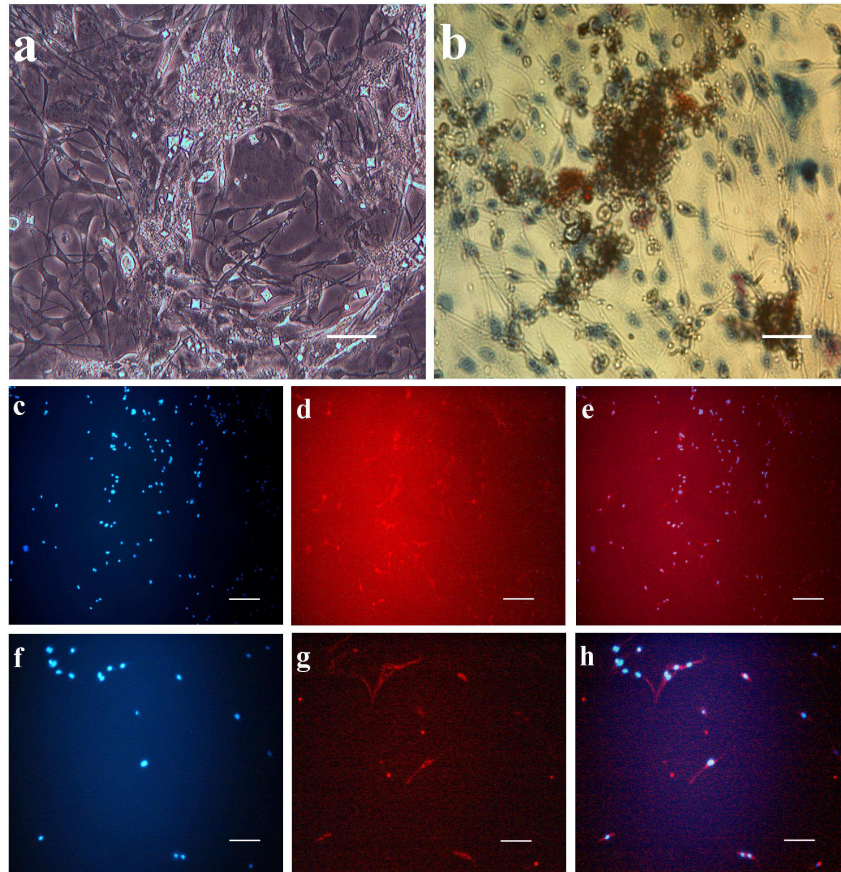


Figure 3. Skeletal Muscle slow-adherent cells characterized by differentiation into Mesodermal lineage. (A) Forming of Calcium-rich crystals from bone differentiation and; (B) Oil Red O for staining of neutral triglycerids with Trypan Blue stain for presentation of vital cells, Skeletal Muscle slow-adherent cells characterized by differentiation into Ectodermal lineage; (C-E) Neural differentiation; (C-D) β tubulin 3 (β tub) and; (C) Cells were co-stained with β tubulin 3 and DAPI for visualize nuclei (blue); (F-G) Microtubule-associated Protein-2 (MAP-2) and; (F) Cells were co-stained with MAP-2 and DAPI for visualize nuclei (blue). Scale bar is 100 μ m

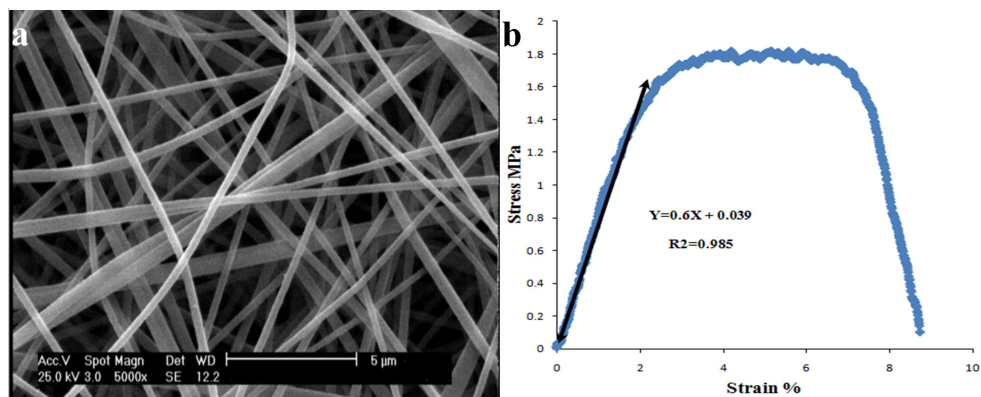


Figure 4. (A) SEM studies of produced nanofibers using electrospinning of PAN; (B) mechanical properties of electrospun PAN membrane

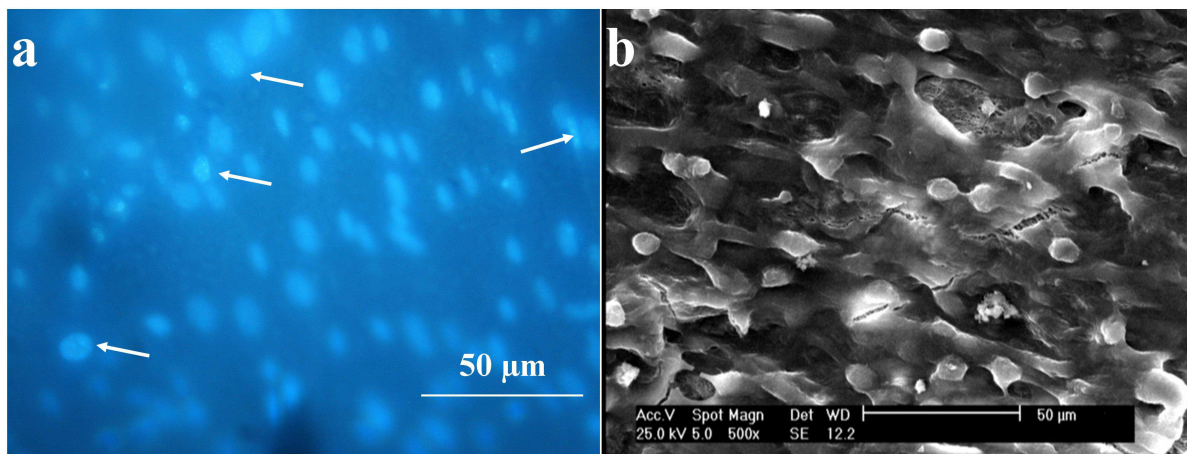


Figure 5. (A) DAPI staining of adhered satellite cells and; (B) SEM examination of extended satellite cells after 15 days of cell culture on electrospun PAN scaffold

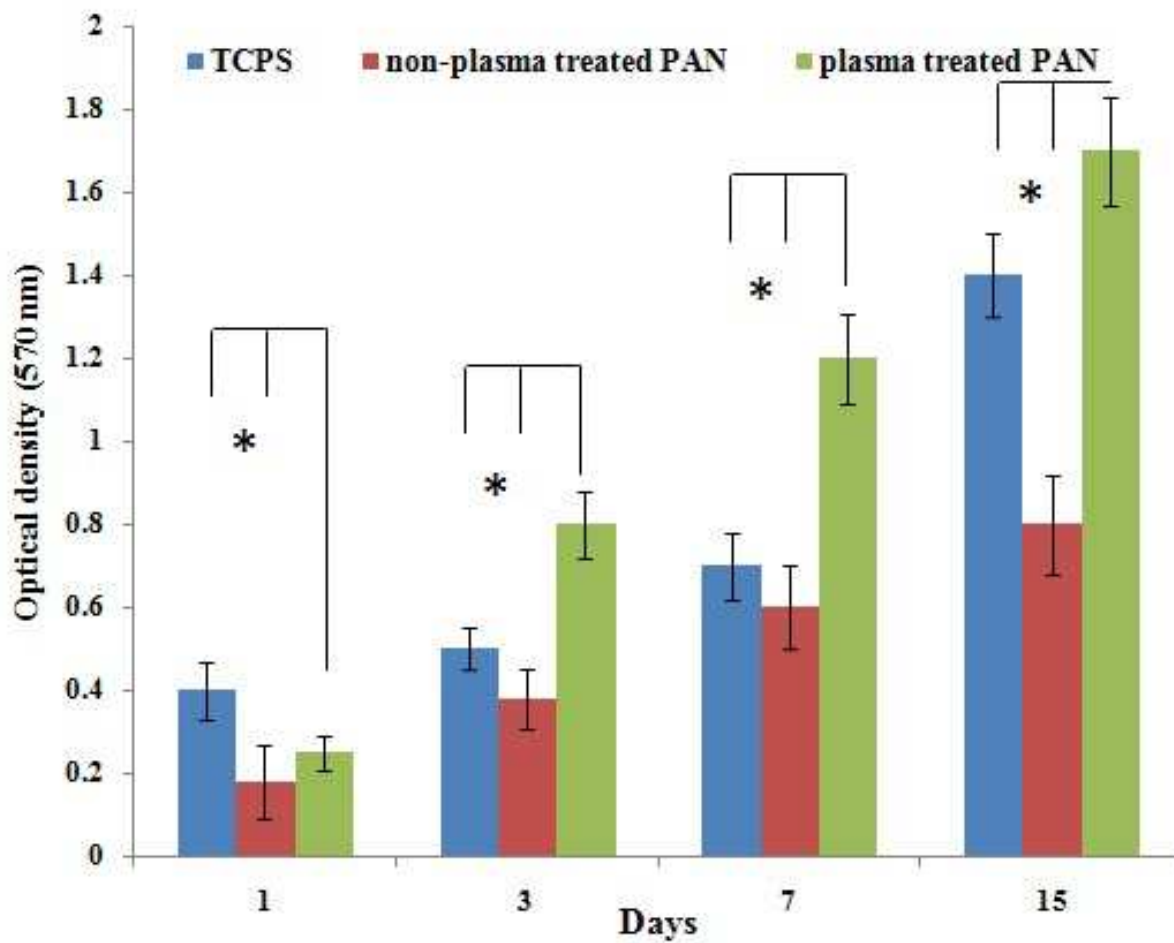


Figure 6. MTT assay of 3 experimental groups including TCPS, non-plasma and plasma treated scaffolds over 15 days of cell culture

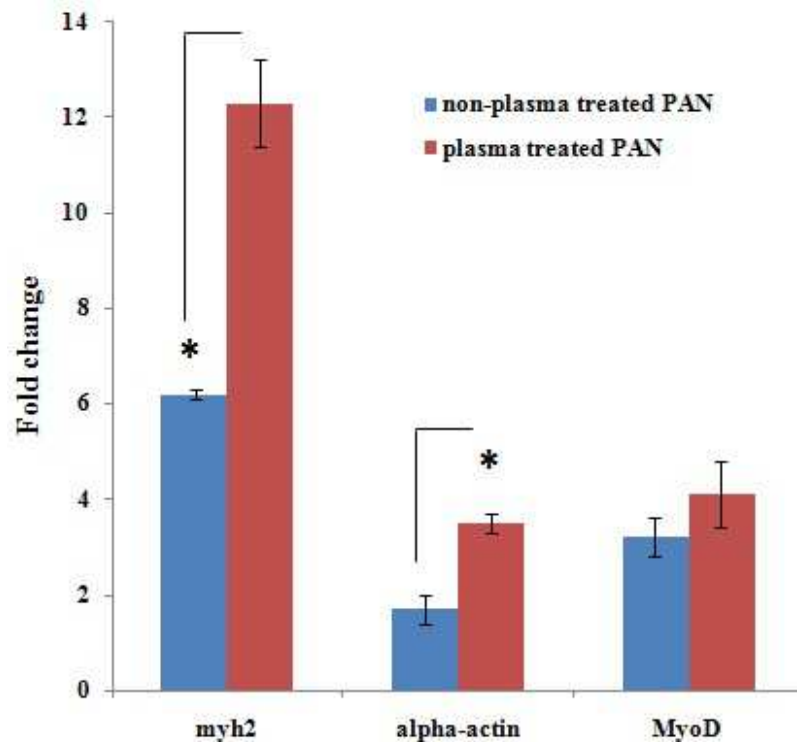


Figure 7. Real-Time PCR assessment to expose the gene expression of cells differentiated on non-plasma and plasma treated scaffolds over 15 days of cell culture.

group (p -value <0.05). The analysis was repeated at 12th day and revealed all markers, except CD34 and α -sarcomeric actin, were down-regulated (p -value <0.050). These results demonstrate pre-plate technique could purify satellite cells and the obtained cells of 6th pre-plating extend to bear more proliferation with a less fluctuation of differentiation markers (MyH and M-cadherin) during *in vitro* cultivation until the 12th day. Absolutely, these cells need more time or using differentiating factors like horse serum for giving rise to multinuclear myocytes. Furthermore, subculture cells as control group had no expression patterns of satellite cell markers after 6 days of cultivation, but they represented the maturation markers after 12 days.

Differentiation of satellite cells to mesodermal and ectodermal lineages

Adult stem cells and progenitors are multipotent, so can give rise to other lineage cells (22). After using the induction media and following staining techniques, we observed their osteogenic, adipogenic, and neuronal differentiation potency. These observations demonstrate the satellite cells are multipotent and capable of forming specific cells as mentioned. However, the differing differentiation ability of

enriched cells had varied between these lineage cells. So the isolated cells were expanded and incubated in an adipogenic medium for 21 days, osteogenic medium for 8 days and neural medium for 6 days. Adipogenic differentiation was weaker than osteogenic lineage by observation of the calcium-rich crystals for osteogenic differentiation and Oil Red O staining for determination of neutral triglycerides (Figure 3 a-b). For neuronal differentiation of these cells, we used the induction media and documented their ability to convert into neural cells by immunostaining of specific markers of neural, microtubule-associated protein-2 (MAP-2) and β tubulin 3 (Figure 3 c-h) (23).

Preparation of PAN nanofibers and examination with SEM

Scaffold preparation of PAN was done at a distance of 17 cm. The applied voltage was relatively 18 kV under ejection value of 0.2 ml/h. The related micrograph of prepared electrospun scaffold from PAN was shown in figure 4. Not only a porous scaffold was obtained by electrospinning method, but also the uniform fiber diameter was also resulted. The fibers showed a regional scale of diameter 743 ± 25 nm which was measured with the Image j software.

Table 1. The list of primer sequences used in this study

Primers	Forward (5'-3')	Reverse (5'-3')	T _m
m-Myh2	GCTTACTTACCAGACAGAAGAAG	TGTTCCCTCAGCCTCCTCAG	62
m-MyoD	CTGATGGCATGATGGATTAC	GACACAGCCGCACTCTTC	62
m- α -sarcomeric actin	CTGATGGCATGATGGATTAC	GCAGACTCCATACCGATAAAG	62
m-cadherin	AGGTGGTGGCTCTCTATAATC	GCCTCCATAAAGAACTCATCC	62
m-CD34	GCCAATAGCACAGAACTTCC	GCCAGCAGAACTCCAGAGG	62
M-Pax7	GCGTAAGCAGGCAGGAG	ATGGTTGATGGCGGAAG	62
Beta-sarcomeric actin as internal control	GTCTCTCCCAAGTCCACAC	GGGAGACCAAAGCCTTCAT	60

The mechanical properties for electrospun PAN

The obtained stress-strain curve of fabricated scaffold was presented in figure 5. The young modulus of PAN membrane was 60 MPa with a linear elastic region up to 2.2%. The break point of corresponding scaffold was relatively 8.6%.

DAPI and SEM examinations of cultured cells on PAN scaffold

DAPI staining was carried out in the following as figure 5a at 15th days of cell culture. The higher cell confluence with plasma treated scaffold was noticeable. Also, the produced some multi-nucleus morphology (the arrows) confirmed the better differentiation potency with seeded satellite cells on plasma treated PAN scaffold. Also, the cell morphology was studied after 15 days of cell culture using SEM (Figure 5b). The efficient cell spreading with plasma treated type of scaffold confirmed ample hydrophilicity after surface modification with plasma treatment. Also, the number of remaining cells on PAN scaffold indicated that the presence of hydrophilic chemical groups was considerable to govern cell adhesion (24).

MTT assays for biocompatibility evaluation of electrospun PAN scaffolds

Further on, the higher optical density of formazan crystals with MTT assay approved higher cell proliferation with this scaffold type at days 1, 3, 7, and 15 for plasma treated electrospun scaffold (Figure 6). There was a significant difference ($P < 0.05$) with a comparison between plasma treated PAN scaffold with both groups of non-plasma treated scaffold type and TCPS that exposed higher grade of biocompatibility with this scaffold.

Gene expression profile of muscular genes for cultured satellite cells on TCPS, non-plasma treated and plasma treated PAN scaffold

The gene expression profile was studied after 15 days of cell culture for non-plasma and plasma treated scaffolds and also TCPS group (Figure 7). The primer sequences are represented

in Table 1 and included the transcription factors (MyoD), contractile apparatus of muscle cells (Myosin Heavy Chain; MyH, α -sarcomeric actin) which were normalized with β -actin (the housekeeping gene of skeletal muscle cells) (25). The expression of muscle genes with non-plasma treated and plasma treated one were calibrated with TCPS gene expression. The all corresponding genes were up-regulated with scaffold groups, though plasma treated PAN scaffold showed significantly higher gene expression value (p value < 0.05) compared to non-plasma treated one for all examined genes except Myo D ($P > 0.05$).

Discussion

The markers of satellite cells have been approximately determined. Transcription factor Pax7 acts as a cell survival signal for maintenance of satellite cells and among the cell surface markers. M-cadherin is expressed in quiescent, activated, and proliferating satellite cells. CD34 is not specific to satellite cells, but has been reported that quiescent satellite cells mostly are CD34 positive. Along these markers, MyoD is compatible with the proliferation and activation of satellite cells (26, 27). Thus increasing of MyoD induces satellite cell differentiation from quiescent form to multi-nuclear cells by giving rise the expression of myosin heavy chain (MyH) and α -sarcomeric actin (28). These markers are useful for characterization of committed and activated satellite cells.

The objective of this study was the assessment of pre-plate technique as a common technique for the enrichment of satellite cells and then utilizing of PAN scaffold to induce cell differentiation efficiently. For this approach, we needed to digestion protocol for a complete dissociation of skeletal muscle tissue to single cells. The protocols were used in the previous studies, could not have suitable efficacy for obtaining in vitro muscle cells. Here, we offer a modified protocol, which is able to digest skeletal muscle tissue as alive and singular cells and the resulted cells had the similar gene expression pattern of satellite cells. This modification was required for improvement



of pre-plate technique, because the enriched cells by using other agents such as Trypsin, PBS or using coated or non-coated conditions by collagen or gelatin were not adequate to purify the cells with the expression profile of satellite cells. By PBS-EDTA, we just transferred the low adherent cells and the gelatin-coated condition was used to complete removing of the adherent cells. In this study, we gradually shift the time of PBS-EDTA from 30 second in the first pre-plating to 4 minutes in the last one. That is because little by little the slow adherent cells are matured and thereupon could earn the stronger adhesive potency.

The gene expression of pre-plated group after 6th step, had the high expression of self-renewal gene, Pax7. On the other hand, M-cadherin is important for fusing the satellite cells to form multi-nuclear cells (29, 30), but has low expression in quiescent phase of satellite cells. In our study, the low-level expression of M-cadherin followed up to 12th day. Furthermore, the low-level expression of CD34 on the 6th stage of the pre-plate technique and its upregulation on 12th day supports the quiescent phase of enriched cells after 2 weeks (13). In contrast, on 6th pre-plate after in vitro cultivation of subculture cells, Pax7 was absent along with the high level of MyH. In our knowledge, satellite cells were removed by further cultivation and the matured muscle cells remained in the control group. Besides of this, in vitro studies of last the pre-plated cells with low potency to differentiate, propose the cells of 6th pre-plate are muscular stem cells (31). Furthermore the ability of differentiation of multiple lineage cells depicts the enriched cells had low ability to adipogenic cells, but with a higher percentage to osteogenic and neural cells. These data support the multipotent potential of isolated cells and their stem cell identity.

Conclusion

For muscle tissue engineering, the PAN electrospun scaffold can provide a nanofibrous surface with higher surface area to volume that guarantees cell satellite cell attachment. The surface modification of corresponding scaffold is most important due to lower adhesion characteristic with satellite cells. Thus, plasma treatment was employed to increase hydrophilic chemical groups with electrospun PAN membrane. MTT assay approved a considerable difference between plasma and non-plasma treated scaffolds. There were higher rate of cell proliferation with plasma treated scaffold type. Also, the formed some multinuclear cells at 15th days of cell culture approved ultimate maturation of satellite cells on plasma treated PAN scaffold. Moreover, the satellite cells, which were cultured on plasma treated scaffold type showed a higher value

of muscle specific gene expression inclusive myh2 and alpha-actin. Myo D exhibited an insignificant relation between plasma and non-plasma treated PAN scaffold.

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Conflict of Interest: 'None declared'.

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