Optimizing Hydrocortisone Concentration for Skeletal Muscles Differentiation of ADSCs on PLLA Nano-Scaffolds

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ABSTRACT

Skeletal muscle is a highly differentiated tissue with very specific functions which has low potential of regeneration. Skeletal muscle injuries especially in athletes almost have lead to muscular dysfunctions and healing may be prolonged for several years. Therefore, working on skeletal muscle differentiation remained an importance in biomedical researches. Adipose derived stem cells (ADSCs) are novel source of mesenchymal stem cells which are an excellent alternative for satellite cells in in-vitro skeletal muscle differentiation. Differentiation potential of ADSCs on both tissue culture plate (TCP) and also on Poly l-lactide acid (PLLA) electrospun fibrous nano-scaffold which now is widely used at tissue engineering investigations has studied in this research. Scanning electron microscopy (SEM) and Tensile test were performed for evaluating scaffold properties. Hydrocortisone has considered a critical factors for skeletal muscle differentiation while, the recommended concentrations of it for inducing myogenesis in stem cells is yet discussing. Statistical analysis of our results from colorimetric MTT assay for various concentrations of hydrocortisone showed that the concentration of 10^{-7} mol/L is the optimum dose for myogenic differentiation of murine ADSCs which was used on both TCP and PLLA scaffolds and skeletal myosin fiber formations was confirmed with immunocytochemistry. DAPI staining proved myocytes nuclei and syncytium formations. Our results also showed that ADSCs and PLLA nano-scaffolds are the suitable biomaterials for engineering skeletal muscle tissue.

Key words: Hydrocortisone; Skeletal Muscle; ADSCs; PLLA; Scaffold

INTRODUCTION

Highly differentiated skeletal muscle cells with distinguished functional specification have low potential for proliferation and regeneration. Therefore, pleasant healing of damaged or defective tissue has been a therapeutic problem [1, 2]. Two main obstacles of stem cell therapy are finding suitable stem cell source and efficient transplantation mechanisms, which are more complicated in case of skeletal muscle tissue. Mesenchymal adipose-derived stem cells (ADSCs) are privileged alternative for low number and unattainable muscle stem/progenitor cells especially in in-vitro culture systems [3].

ADSCs are highly proliferative and can be adequately obtained by noninvasive surgery. Excellent differentiation potential of these cells to various tissues and their immunemodulatory features have demonstrated in recent published works [4, 5]. Hydrocortisone mentioned as critical factor of skeletal muscle differentiation in most articles [6, 7] while the suitable concentration of it is not scientifically determined yet. Hydrocortisone concentration mentioned to make various responses in different cell types and can cause growth inhibition and aging at in vitro cell cultures [8, 9].

Therefore, determination an optimum concentration of it for skeletal muscle differentiation should be well investigated.

Poly L-Lactide Acid (PLLA) is a biodegradable and biocompatible polymer that breaks down to safe material of lactic acid. PLLA nanofibers that now are widely using in tissue engineering can be easily obtained by electrospining method of scaffold fabrication [10-12].

In this study we tried to differentiate murine ADSCs to skeletal muscle cells by various concentrations of hydrocortisone and then the cells viability, proliferation and differentiation was examined on PLLA fibrous scaffold.

MATERIALS AND METHODS

Electrospining and Mechanical properties of the PLLA Scaffold

As it previously described for PLLA electrospining method [13-15], the polymer of poly l-lactide acid (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) with MW of 240,000 g/mol was dissolved in chloroform (Merck, Germany) and dimethylformamaide (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) (4.25:0.75) and was remained on stirrer for about 3h.

The obtained material was loaded to plastic syringes with 21-gauge needle that was placed at 15 cm from collector which was set to rotate with 2300 rpm. Nanofibers were collected by applying positive voltage of 18 KV between needle and collector. Oxygen plasma surface modification performed for 5 min by frequency plasma generator (Diener Electronics, Germany) to make the scaffold hydrophilic. Before cell seeding, the scaffolds were sterilized by ethanol 70%. Tensile tester (SANTAM stress machine, Iran) at speed rate of 50 mm/min was utilized to examine mechanical aspects of fibrous PLLA scaffold pieces that were cut in 60×10 mm^2 rectangular.

Scanning Electron Microscopy (SEM)

The PLLA electrospun nanofibrous scaffolds simply were gold sputtered in vacuum and AIS2300C scanning electron microscope (SERON, Korea) was used for imaging.

Cell Culture

Murine ADSCs at first passage were purchased from Stem Cell Technology Research Center (Tehran, Iran) and were expanded. Second passage cells were cultured in 24 well plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin (all from Gibco, USA) to reach 90% confluency. The plates were incubated in humid incubator at 37°C and 5% CO2. Then, myogenic differentiation medium contained DMEM supplemented with 10% FBS, 10% horse serum with various concentrations of hydrocortisone include 0, 5×10^{-5}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9} (mol/L). The ADSCs were also cultured on O_2 plasma treated and sterilized PLLA scaffolds which were punched to fix in 24 well cell culture plates at the density of 1×10^4 per well. After 24h (for allowing cells to adhere), ADSCs were differentiated using 1×10^{-7} concentration of hydrocortisone at the myogenic differentiation medium.

MTT Assay

To study the effect of hydrocortisone concentrations on the cells viability colorimetric assay was performed. Mitochondrial activity of viable cells was measured by 3-(4,5-dimethythiazol-2-yl) 2,5diphenyl tetrazolium bromide (MTT) assay.

The blue crystals of formazan were dissolved in dimethyl sulfoxide (DMSO) and the optical density of solution read at 570 nm using BioPhotometer (Eppendorf, Germany). The test was performed at 2 and 4 days after applying differentiation medium. After determination of best hydrocortisone concentration, MTT assay was also performed for ADSCs were cultured on PLLA scaffold in comparison with tissue culture plates during 5 days with and without applying myogenic differentiation medium.

Immunocytochemistry

The expression of myosin at the differentiated cells on PLLA scaffolds and normal culture was investigated by applying a routine protocol of Immunocytochemistry.

The Monoclonal anti-myosin (skeletal, slow) Mouse Ascites Fluid (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com); (2:1000) and Goat F antimouse IgG (PE) (Cedarlane, Hornby, Ontario, Canada); (2:1000) were used as the first and secondary antibodies, respectively. DAPI (4',6-diamidino-2-phenylindole) staining was done for nuclear staining. Fluorescence was analyzed using an inverted fluorescence microscope (Nikon TE-2000, Japan).

Statistics

Two-way analysis of variance (ANOVA) and bonferroni post-test were used for statistical analysis of quantitative results that were expressed as mean ± standard deviation (SD) and $P<0.05$ considered statistically significant.

RESULTS

Scanning Electron Microscopy

The SEM images indicate the obtained random PLLA fibrous scaffold using electrospining method (Figure 1).

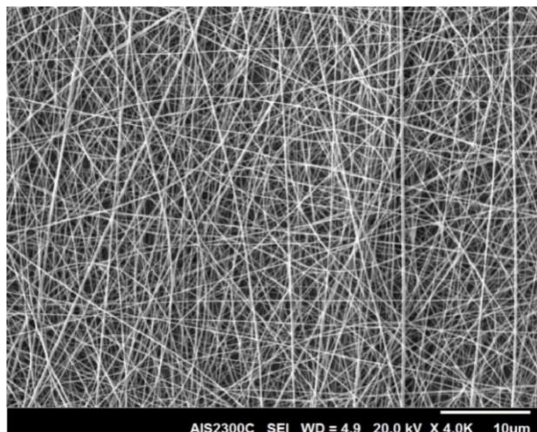

Figure 1. SEM image of PLLA nanofibrous scaffold fabricated by electrospining method. The scale bar is 10 µm.

Mechanical Properties of PLLA Scaffold

Stress-strain curve indicates plasticity and mechanical properties of the scaffold which provided proper aspects for skeletal muscle tissue engineering (Figure 2).

Cells Proliferation and Viability

Figure 3A shows the statistical analysis of MTT results which compares viability of murine ADSCs treated by myogenic differentiation with various concentrations of hydrocortisone at day 2 and day 4. Graphs indicates the amounts of cells viability at the hydrocortisone concentrations less than 10^{-7} mol/L was much lower than 50% amount of cells viability at the concentration of 0. Figure 4c displays cell death that occurred by using concentration of 1×10^{-6} mol/L hydrocortisone in the differentiation medium at day 5 while the concentration of 1×10^{-7} has induced differentiation without decreasing the cells number in comparison with control (Figure 4a and b). Even without staining the aggregations of the nuclei at the myocytes can be detected at figure 4b.

However there was no significant ($P>0.05$) differences among day 2 and 4 at concentrations of 1×10^{-7} and 1×10^{-8} mol/L, but the cells viability was higher at the day 4 versus day 2 at the concentration of 1×10^{-8} mol/L. Reversely, the cells were still proliferative at 1×10^{-6} mol/L. Also, the results indicated that the proliferation of the cells at the concentration of 1×10^{-9} did not much differ from culture without hydrocortisone (concentration of 0) at the both time points. Interestingly, it seems that cells proliferation has stopped at the concentration of 1×10^{-7} mol/L considering this dose was not lethal (the cells viability was more than 50%).

MTT assay also performed to compare cells proliferation and viability between normal (TCP) and 3D cultures (Figure 3B). Statistically analyzed data showed that the cells proliferation and viability was significantly ($P<0.05$) higher at the 3D culture against TCP after 4 days. In case of myogenic medium that the cells were at 90% confluency at day 0, it seems that however the viability of cells was significantly ($P<0.05$) higher at 3D culture but the viability amount was remained almost constant at both TCP and 3D culture (Figure 3C).

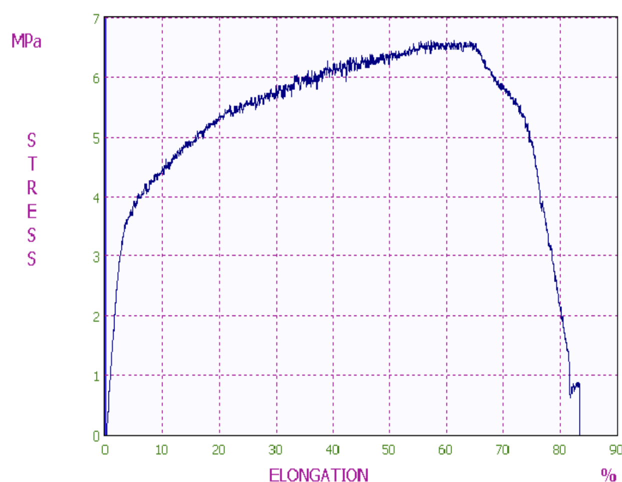

Figure 2. Stress-strain curve of PLLA nanoscaffold.

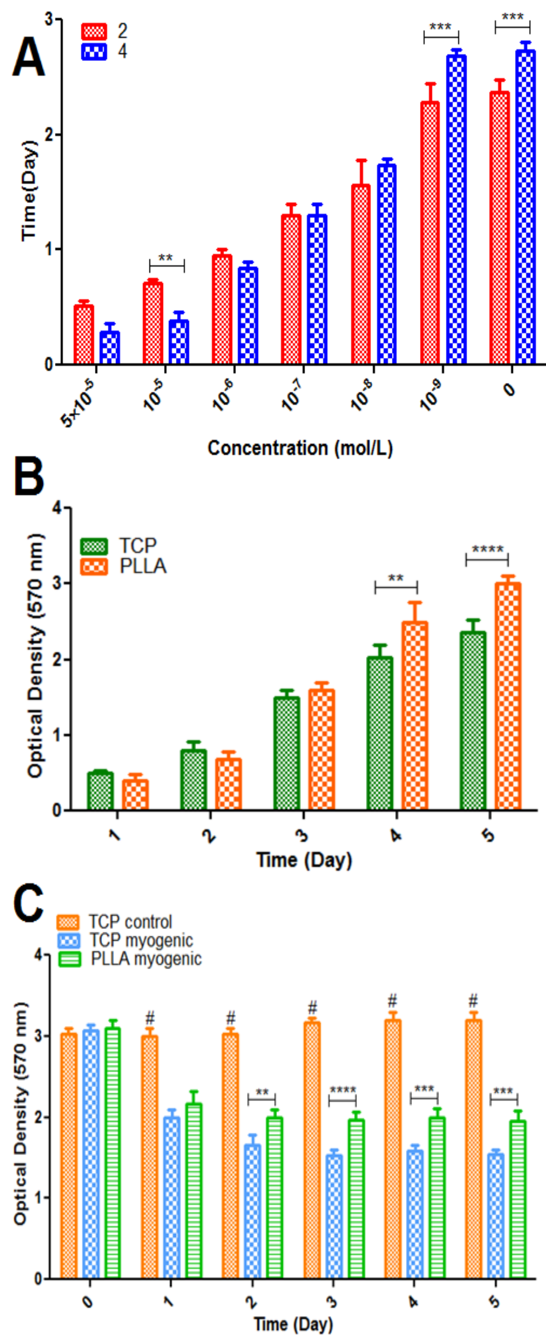

Figure 3. Statistical analysis of MTT results; (A): comparing various concentrations of hydrocortisone at the myogenic medium on ADSCs at day 2 and 4. (B): comparing the ADSCs proliferation between TCP and on PLLA scaffolds during 5 days. (C): comparing cells viability among TCP cultures and on PLLA scaffolds during 5 days of applying myogenic medium (the TCP control stands for cells on normal culture medium); the cells were at the 90% confluency before treating. N=3, Mean±SD. $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$.

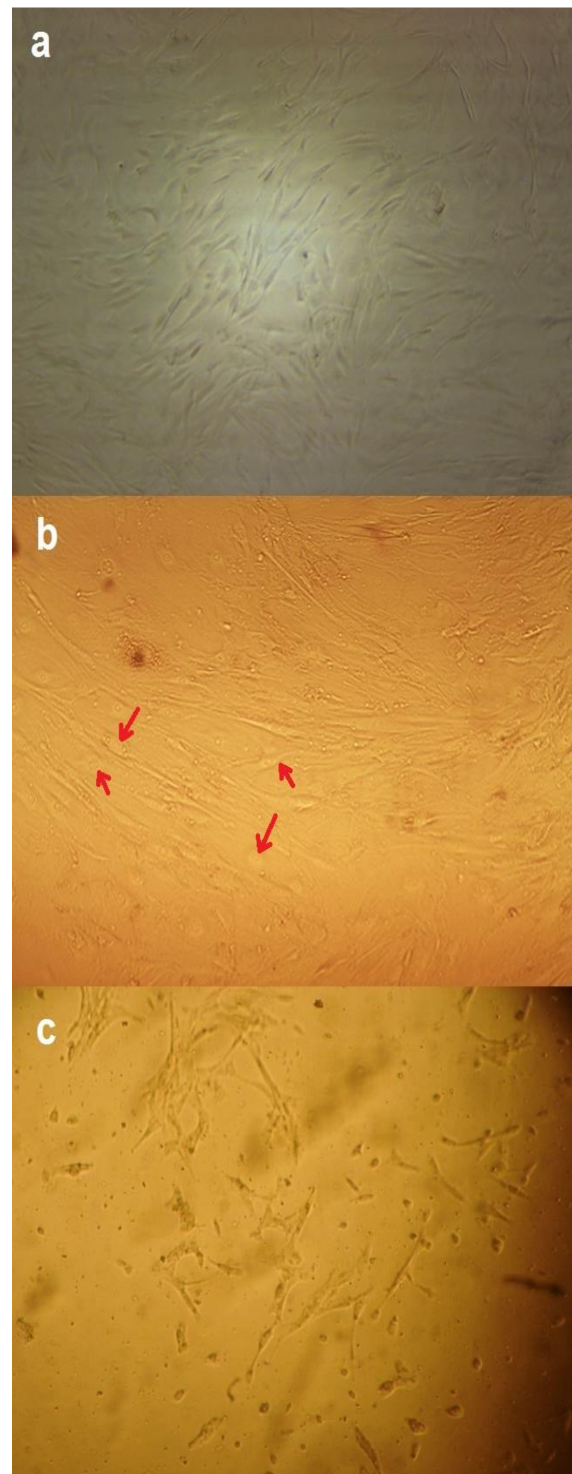

Figure 4. Shows the ADSCs cultured in normal culture medium (a) and in skeletal cells differentiation medium with concentrations of 10^{-7} (b) and 10^{-6} (c) mol/L of hydrocortisone at day 5 post-differentiation. 4X. Arrows indicate aggregations of nuclei at myocytes.

Cell differentiation

The formation of myosin fibers at the differentiated myocytes and myotubes is showed by immunofluorescent staining at the normal and 3D cultures on PLLA nanofibers. Nuclear staining by DAPI represented the cells and Syncytium formations during myotube development (Figure 5).

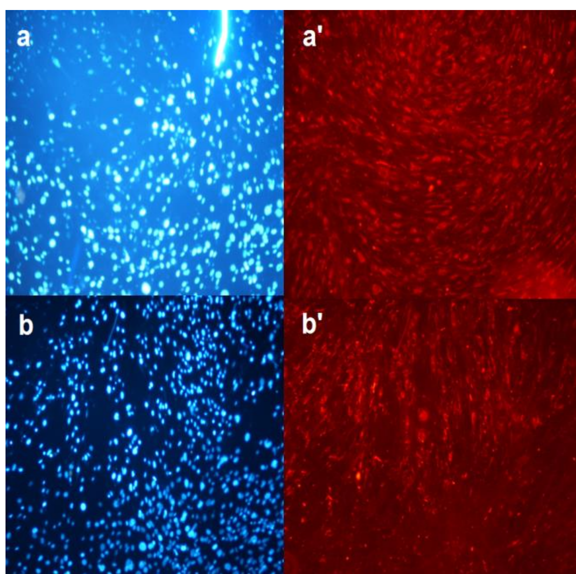

Figure 5. Immunocytochemistry of differentiated skeletal myocytes at 3D culture on PLLA scaffold (a, a') and TCP culture (b, b'). Nuclei were stained DAPI (a and b) and the myosin fibers formation (a' and b') in differentiated murine ADSCs are shown.

DISCUSSION

Fibrous structure of PLLA scaffold as it showed by SEM images can provide better environment for cells to nourish, growth and proliferate. Statistical analysis of MTT results represented that after 3 days of the culture the proliferation and viability of ADSCs were significantly ($P<0.05$) increased on 3D culture compare to TCP. The PLLA polymers are also biocompatible and did not affect cells growth; the proliferation of ADSCs significantly ($P<0.05$) increased on PLLA scaffold after 4 days of culture. Therefore, as it previously described in authors reports, PLLA has remarkably improved the quality of the cell's environment [14, 16-18]. Results of tensile test also has indicated that the scaffold has suitable mechanical properties and plasticity for skeletal muscle differentiation as it mentioned by researchers [19, 20]. There is a lack of determining the exact concentration of hydrocortisone which induces skeletal muscle differentiation in mesenchymal stem cells. Different studies have suggested various concentration of hydrocortisone to use in skeletal muscle differentiation medium; a range of 18 mg/mL [21], 0.2 μM [22], 50 μM hydrocortisone [23, 24] and even 2% [25] to 5% hydrocortisone [26] have been noted. While, the statistical analysis of our MTT results from various hydrocortisone concentrations in TCP cultures demonstrated that concentration of 1×10^{-7} mol/L is suitable dose for inducing skeletal muscle differentiation which stops the cells proliferation and is not toxic while the higher concentrations of 5×10^{-5} , 1×10^{-5} and 1×10^{-6} mol/L seems to be more lethal for mesenchymal stem cells than inducing differentiation; at those concentrations of hydrocortisone the rate of cell death is more than 50% in comparison with medium free from hydrocortisone. Lower doses of 1×10^{-8} and 1×10^{-9} mol/L did not fully stop the cells proliferation and did not much differ from control group (medium without hydrocortisone). Analysis of MTT results from 3D and TCP cultures by applying myogenic differentiation with concentration of 10^{-7} mol/L of hydrocortisone also illustrated that scaffold did not interfered or affected skeletal muscle differentiation as it confirmed by ICC results that has stained the myosin fibers which were well generated at both culture types.

Consequently, ADSCs and PLLA nanoscaffold are the correct biomaterials for skeletal muscle tissue engineering and the concentration of 10^{-7} mol/L of hydrocortisone is suggested as the optimum dose for skeletal muscle differentiation of murine ADSCs. Myogenic differentiation protocols and techniques yet need to be improved by further researches.

CONCLUSION

In this study we compared various concentrations of hydrocortisone for inducing myogenic differentiation and the concentration of 10^{-7} mol/L was selected to be the most suitable dose for skeletal muscle differentiation of murine ADSCs. The PLLA nanofiber electrospun scaffold also introduced to has excellent performance for supporting myogenic differentiation of stem cells.

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