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Cytoprotection, proliferation and epidermal differentiation of adipose tissue-derived stem cells on emu oil based electrospun nanofibrous mat

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ABSTRACT

Electrospun nanofibrous scaffolds containing natural substances with wound healing properties such as Emu oil (EO) may have a great potential for increasing the efficiency of stem cell-based skin bioengineering. For this purpose, EO blended PCL/PEG electrospun nanofibrous mats were successfully fabricated and characterized using FE-SEM, FTIR and Universal Testing Machine. The efficiency of the scaffolds in supporting the adherence, cytoprotection, proliferation and differentiation of adipose tissue-derived stem cells (ADSCs) to keratinocyte was evaluated. GC/MS and HPLC were used to determine the composition of pure EO, which revealed to be mainly fatty acids and carotenoids. FE-SEM and cell proliferation assays showed that adhesion and proliferation of ADSCs on EO-PCL/PEG nanofibers was significantly higher than on PCL/PEG nanofibers. Additionally, EO-PCL/PEG nanofibers with free radical scavenging properties conferred a cytoprotective effect against cell-damaging free radicals, while the ability to support cell adhesion and growth was maintained or even improved. Immunostaining of ADSCs on EO-PCL/PEG nanofibers confirmed the change in morphology of ADSCs from spindle to polygonal shape suggesting their differentiation toward an epidermal linage. Moreover, the expression levels of the keratin 10, filaggrin, and involucrin that are involved in epidermal differentiation were upregulated in a stage-specific manner. This preliminary study shows that EO-PCL/PEG nanofibers could be a good candidate for the fabrication of wound dressings and skin bioengineered substitutes with ADSCs.

1. Introduction

Skin is one of the highest proliferating tissues of the body and this tissue is usually able to restore small partial thickness wounds. However, large full-thickness injuries, burns and traumatic wounds considerably hamper the self-healing process of skin [1]. These injuries need an appropriate coverage, in order to accelerate healing and aid tissue repair. Autologous split-thickness skin graft is the clinical gold standard to treat major wounds, but the availability of healthy skin is restricted and it causes donor-site injuries [2]. While allografts are another solution, they present the risk of transmitting infectious agents and cause immune rejection [3]. Recent advances in fabrication of tissue-engineered skin grafts include the use of bioactive scaffolds with dermal fibroblasts or epidermal keratinocytes [4]. Culturing the cells *in vitro* and preparing such scaffolds are time-consuming and this strategy is not well adapted for emergency clinical demands. Thus, the existing treatment approaches to treat large skin wounds do not meet the clinical needs [5].

Human adipose–derived stem cells (ADSCs) have been recently recognized as a main cell source for tissue engineering applications, because of their ease of isolation from subcutaneous adipose tissue, extensive proliferation ability, and hypoimmunogenic nature [6,7]. It has been shown that ADSCs dynamically contribute in the different stages of wound healing process through secreting various growth factors and anti-inflammatory cytokines, encouraging re-epithelialization and neo-vascularization, and furthermore by regulating fibroblasts' phenotype and extracellular matrix (ECM) deposition [8–10]. In addition, recent reports have shown that delivering ADSCs reduces scarring, while the mechanisms have not been fully clarified [11,12]. Because of their excellent proliferation and differentiation potential, ADSCs became a key factor in cell-based skin bioengineering.

Although stem cell therapy in the skin have shown a variety of benefits in both pre-clinical reports and clinical trials, poor cell survival after transplantation continues is the main issue [13]. This is princi-

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pally attributed to the inflammatory and oxidative stress environment at the site of injury [14]. It has been suggested that endogenous antioxidant levels of stem cells could affect their fate following transplantation at damaged sites. Similarly, it seems that after treatment with antioxidants, mesenchymal stem cells (MSCs) show increased survival ratio and better regenerative effects [13].

There is now a variety of skin grafts for clinical application and the biomaterials commonly applied as artificial ECM comprise naturally-derived materials (e.g. collagen and hyaluronic acid) and synthetic materials (e.g. Poly-caprolactone and poly-glycolic acid) [15]. However, there are currently no biomaterials that fully simulate the anatomical, physiological, and biological characteristics of healthy skin. Some issues, among others, including poor wound healing, weak vascularization, and the lack of differentiation factors often arise [16,17]. Therefore, viable and safe substitutes are still required. Electrospun nanofibrous scaffolds containing bioactive molecules with wound healing properties may have a great potential in cell-based skin bioengineering and solve these problems. Electrospun nanofiber scaffolds provide appropriate molecular signaling, when merged with or/and immobilized with bioactive molecules in 3D structure [18]. Various bioactive molecules such as growth factors, vitamins, antibiotics, analgesics, anti-inflammatory drugs, as well as herbal extracts and recently animal fats with wound healing proprieties have been incorporated into nanofiber scaffolds. The proliferative and antibacterial properties of such scaffolds were assessed [19,20], but few studies have evaluated the cytoprotective and differentiation effects of the bioactive scaffolds.

Animal fats such as Emu oil (EO) have been applied traditionally as wound healer and the native Aboriginals and first settlers in Australia used the oil to accelerate the wound healing process and to relieve pain. EO was also used for various musculoskeletal injuries or disorders [21,22]. Several *in vitro* and animal studies suggest that EO treatment can promote re-epithelialization in wound areas and may have antioxidative, anti-inflammatory and antibacterial properties [23,24].

In the present study, we have evaluated the efficiency of EO-PCL/PEG electrospun nanofibrous scaffolds in supporting the adherence, proliferation, cytoprotection and differentiation of ADSCs into keratinocytes. In addition, we have investigated the antioxidative effects of EO-based nanofibers on ADSCs undergoing oxidative stress. EO-PCL/PEG nanofibrous scaffolds captured with ADSCs have the potential to be efficient bioengineered cell–scaffold constructs for skin tissue regeneration and also provide an immediate coverage to deep wounds, burns and posttraumatic wounds.

2. Materials and methods

2.1. Materials

ε-caprolactone (ε-CL) and polyethylene glycol (PEG, Mn=4 kDa), hydrocortisone, insulin, T3, EGF, VD3, L-ascorbic acid and MTT was purchased from Sigma-Aldrich (U.S.A). Dichloromethane (DCM, 99.5%) and Methanol (99.0%) were purchased from Merck Chemical Co. Dulbecco's Modified Eagle's Medium (DMEM), DMEM/F12, fetal bovine serum (FBS), Penicillin/Streptomycin, and trypsin were purchased from Gibco (Invitrogen, NY, USA). All the chemicals were used without further purification.

2.2. Fatty acid composition analysis

We examined 3 samples of emu fats that were supplied by an emu farm in Azerbaijan, Iran. The fats were harvested from the main fat depots from the intra-abdominal (retroperitoneal) and subcutaneous areas of emu and frozen and stored at −20 °C. For rendering the fat into oil, first, the fat was thawed, ground or minced, and heated to around 60–70 °C for 30–60 min. The obtained oil was filtered and stored in nitrogen to avoid oxidation. For GC/MS analysis, total lipids were extracted by chloroform: methanol (2:1 v/v) containing 0.01% butylated hydroxyl toluene as the antioxidant, and changed to fatty acid methyl esters by transesterification with boron trifluoride in methanol. After extraction with hexane, fatty acid methyl esters were analyzed by a Shimadzu QP-2010 Plus gas chromatograph mass spectrometer (GC/MS) armed with an AOC 20i+s auto injector. An Rtx®-5MS 30 m×0.25 mm ID×0.25 μm column was applied for the analysis. The injector was in splitless mode (1 min hold) with 1 μL injection volume at an inlet temperature of 280 °C. Helium was applied as the carrier gas set at constant flow 0.9 mL/min. The oven schedule had an initial temperature of 50 °C, then immediately ramped at 5 °C/min. to 260 °C (hold 10 min). Chromatographic peaks were integrated and identified applying the Shimadzu software package (version 7.2.1 SP1). Individual fatty acids were reported as weight percent of total fatty acids.

2.3. Carotenoid analysis

EO samples were homogenized with an equal volume of ethanol containing 0.1% butylhydroxytoluene (BHT) as an antioxidant. The carotenoids were extracted with hexane/ethyl acetate (90/10, v/v), and evaporated under stream of nitrogen. before injection, the residue was dissolved in ethyl acetate, diluted in mobile phase, vortexed, and sonicated. Chromatographic measurements were preformed applying a Thermo Separation Products HPLC system (San Jose, CA) including a solvent degasser, an autosampler (AS3000) maintaining samples at 20 °C, a 3-Nm column (Spherisorb ODS2; 4.0×250 mm with titanium frits), a column heater at 31 °C, a time-programmable UV/visible detector (UV 2000), a programmable dual Monochromator fluorescence detector (FL2000), and a computer data system (PC1000). The separation was carried out isocratically applying a mobile phase of acetonitrile: methanol: propanol (40:50:10) at a flow rate of 0.8 mL/min. Carotenoids were detected by absorbance at 450 nm.

2.4. Fabrication of nanofibrous mats

First, PCL/PEG (PCL:PEG=90:10, w/w) copolymers were synthesized by ring-opening polymerization of ε-CL initiated by PEG and $Sn(Oct)_2$ as a catalyzer and then, PCL/PEG copolymers were dissolved in DCM: methanol (4:1 *v/v* ratio) to produce a solution of 10% *w/v* concentration. For the preparation of EO-loaded PCL/PEG solution, 20 wt% of EO with respect to the PCL/PEG content were added to PCL/PEG solution and stirred magnetically at room temperature for 8 h at 25 °C. Carefully, EO was added dropwise to the polymer solutions and kept for stirring for 1 h prior to electrospinning. Each of the prepared solutions was placed into a standard 5 mL plastic syringe with a blunt-ended stainless steel hypodermic needle tip (gauge 22). Electrospun nanofibrous mats were collected on a rotating collector covered with an aluminum foil. All the electrospinning processes were performed at a range of 27–30 kV, a 200 mm needle tip to collector distance, and a 2 mL/h solution flow rate. The mats were dried in a vacuum dryer for 24 h at room temperature to remove the residual solvent, and this sample was applied for further characterizations.

2.5. Characterizations

The morphology of the electrospun fibers was characterized using field emission scanning electron microscopy (FE-SEM) (MIRA3 TESCAN, Czech) at 25 kV. The average diameter and distribution of the nanofibers were determined from the FE-SEM photographs using image analysis software (Image J, National Institutes of Health, Bethesda, VA). The bonding configurations of the electrospun nanofibers were characterized using a Shimadzu 8400 s Infrared Spectrophotometer (Kyoto, Japan).

The samples were carefully cut into 10 mm×50 mm rectangular strips, and their mechanical properties were measured using a