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Study of epithelial differentiation and protein expression of keratinocyte-mesenchyme stem cell co-cultivation on electrospun nylon/*B. vulgaris* extract composite scaffold

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ABSTRACT

Employing of the composite electrospun scaffold containing herbal extract in conjugation with co-culturing of cells can open up new window to the design of efficient biomaterials for skin tissue regeneration. Here, we introduce the synergistic effect of composite electrospun nanofibrous scaffold of nylon66 loaded with *Beta vulgaris* (*B. vulgaris*) (extract of beet roots, a plants whose widely used in Iranian folk medicine as wound healing medicine) and co-culture of mesenchymal stem-cells (MSCs)-human keratinocyte (H-keratino) differentiation towards epithelial lineage. *In vitro* biocompatibility was examined through MTT assay and epithelial differentiation checked by real-time PCR and immunocytochemistry (ICC) assay after co-culturing of MSCs and H-keratino on proposed scaffold. Significant enhancement in cell proliferation was detected after cell culturing on the composite type of electrospun scaffold containing *B. vulgaris*. Moreover, after 14 days of co-culturing process, gene expression results revealed that both composite and non-composite nylon66 electrospun scaffold promote epithelial differentiation compared to mono-cell culturing of H-keratino in terms of several markers as Cytokeratin 10, Cytokeratin 14 and Involucrin and ICC of some dermal proteins like Cytokeratin 14 and Loricrin. To the best of our knowledge, findings of this study will introduce new way for the generation of novel biomaterials for the development of current skin tissue engineering.

1. Introduction

Skin is the largest organ in the body which protects it from the penetration of toxic substances, pathogens, and organisms. Moreover, it provides the body a pathway for the transport of active reagent like drugs. Skin defects caused by burn, accident, chronic wounds, and diseases, *etc.* can interfere with skin functions as a barrier for body and based on the wound severity it can lead to permanent disability or death [1]. Throughout the world, skin grafts are in high demand as the best way for skin treatment and included 50% of tissue engineering and regenerative medicine market revenues. For example, it was reported in 2009, the potential market in United States for skin replacements and substitutes are approximately $18.9 billion for patient population of approximately 5.0 million and it is prospected that by the year of 2019, these population increase to 6.4 million and result in a potential US market of approximately $24.3 billion [2]. Therefore, as it can be concluded, there is an increasing demand for artificial skin.

In the native environment, natural tissues in the body often comprises of two or more types of cells which interact with each other to facilitate viability, proliferation [3,4] as well as secretion of necessary growth factors and proteins [5,6] to enhance differentiation [7]. For example, blood vessels are consist of the multi-cellular system of endothelial cells, vascular smooth muscle cells, and fibroblasts [8]. In some cases, on the influence of a disease or injury, some native tissues, like skin, do not have ability to regenerate themselves or, sometimes, the body try to repair damages but often the repaired tissues do not have the same functionality of the original tissue [9]. Therefore, in such cases, tissue

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engineering approaches have attracted great interest to regenerate damaged tissues and organs. During last decades, most of studies have focused on mono-cell culturing studies for regeneration of special tissue. However, recent studies have increasingly found their way to use co-culturing of different cell types in tissue engineering as these systems have inherent potential to better model physical and biological parameter of the natural tissues. Biochemical and biophysical structure of microenvironment and intercellular interactions of different cell types play a crucial role in skin regeneration. Human skin consists of the epidermis and dermis which the epidermis being the outer layer of the skin. The major cells of the epidermis are keratinocytes (90–95%), which proliferate in the *stratum basale* (the deepest layer of epidermis) [10]. It was well known that multipotent mesenchymal stem cells (MSCs) have capacity to differentiate into mesenchymal lineage cell including chondrogenic, osteogenic and adipogenic lineages [11]. Moreover, it is also reported MSCs can differentiate to neuroectodermal, mesodermal, and endodermal lineages through intercellular interaction between mature cells *i.e.* by means of co-cultured systems. For example, It is reported that by non-contact co-cultivation of keratinocytes and hMSCs, MSCs can differentiate into early myofibroblasts and neural lineages, while MSCs will differentiate towards epithelial linage in contact co-culture system [12].

Recently, nanofibrous scaffolds have been introduced as an appropriate substrate for tissue engineering of damaged skin. The corresponding scaffolds should provide enough mechanical, physical and chemical property to mimic native skin tissue [13,14]. Varying techniques have been reported for preparation of nanofiber that among them, electrospinning has been widely used as an economical and high throughputs technique for production of ultrafine fibers from wide range of biodegradable polymers [15,16]. Electrospun nanofibrous membrane formed by electrospinning which have similarity to natural extracellular matrix (ECM) may be the most promising types of scaffolds for tissue engineering application [17]. The nano-sized features of a given scaffold could play a crucial role for simulation of ECM structure of different type of tissues. Base on the collector type in electrospinning, nanofibrous membrane with different alignment can be produced to mimic ECMs structure of a desired tissue. These scaffolds can guarantee transport of nutrient supply and oxygen for cellular growth that are required for successful skin regenerative amis.

To some extent, herbal materials containing polysaccharide ingredient have been reported to treat skin tissue [18]. Hence, a nanofibrous mat which is incorporated by polysaccharide derivatives as bioactive agents can accelerate wound healing. *B. vulgaris* is an extract which obtained from the roots of beet (Chenopodiaceae or Amaranthaceae) a plants whose powders of roots widely used in Iranian folk medicine on the basis of Iranian Traditional books as a relief for skin wounds [19]. Beet is found throughout the west and north of Iran with the local name of “Cheghondar”. The chemical composition of the beet roots is mainly from to polysaccharides group specially pectin which is widely used for the skin treatment injuries in Iranian folk medicine on the basis of Iranian Traditional books [20]. On the other hand, skin consist of the high ratio of protein namely elastin with the high degree of elasticity hence, in order to better mimic skin structure [21], a scaffold construct from a polymer with ample elastic properties may be an appropriate candidate for skin reconstruction. In this manner, nylon66 (polyamide polymer) with minimal inflammatory reaction, may be an excellent candidate for skin tissue engineering. Therefore, nylon incorporated in *B. vulgaris* can provide adequate elasticity for freely skin movements.

Based on this finding, we are interested to investigate, for the first time, the synergetic effect of nylon-*B. vulgaris* nanofibrous membrane (N-B.v NFM) as scaffold and co-cultivation of MSCs/keratinocyte cell on the epithelial differentiation of MSC cells for skin tissue engineering applications. As it is known in addition to initial adhesion and proliferation of MSCs, their differentiation to epithelial lineage is a key parameter for skin regeneration. Physiochemical and mechanical characteristics of designed scaffolds were assessed through SEM, contact angle, tensile test, and NMR. The cell response and keratinocyte activity of MSC/H-keratino cultured on different scaffolds with and without *B. vulgaris* were analyzed through MTT, SEM, real-time PCR, DAPI and immunocytochemistry (ICC).

2. Materials and methods

2.1. Preparation of the B. vulgaris extract

100 g fresh powder of the beet roots was added into 300 ml of HCL (Merck) 1% and heated under reflowing condition for 1 h. Resulting solution was cooled down and transferred into cotton cloth to sieve with hand pressure. Then, 300 ml of ethanol (Merck) was added into obtained solution. The sediment was separated and dissolved in water and condensed again using ethanol. Clarity - Chromatography SW was used to find the main composition of extract. The injection volume was 20 μl. The mobile phase consisted of HPLC-grade water-acetonitril. Flow rate was set to 1 ml/min, and the wavelength of 200 nm was applied.

2.2. Synthesis and characterization of N-B.v NFM

Formic acid was used as solvent for preparation of nylon solution with the concentration of 12% (w/v). Nylon was dissolved in formic acid and stirred for 2 h at room temperature. After the preparation of clear solution of nylon/formic acid, obtained extract was added to the prepared solution with the ratio of 50/50 (w/w). The solution was stirred again for 24 h at room temperature.

2.3. Electrospinning process

The prepared solution was loaded into a 22-gauge needle through an extension tube by a syringe pump connected to a plastic syringe. The speed of rotating collector was adjusted to 300 rpm and a high voltage of 19 kV was applied between nozzles and collector through DC high-voltage power supply (Stem cell Technology Research Center, Iran). The flow rate of polymer solution was adjusted to 0.2 ml/h. The needle was connected to the positive electrode of high-voltage supply. The distance between the needle tip and the rotating collector was set to 12 cm. The entire electrospinning process was carried out at room temperature. The electrospinning process was continued with two syringe nozzle for 3 h to obtain the desired thickness of the composite nanofiber.

2.4. Scaffold characterization

Philips, XL-30 scanning electron microscope, was used to observe the surface morphology of the electrospun composite and non-composite nanofibrous membrane at an accelerating voltage of 26 kV under magnification of 5000×. Average fiber diameter was observed from five SEM images.

The hydrophilicity of the prepared scaffolds was assessed by sessile drop method. For this purpose, contact angle of a water droplet on the surface of scaffolds was measured through an optical bench type contact angle measuring system (Rame-Hart instrument company, Model 100-0, USA).

Scaffold's mechanical property was analyzed with a 50 mm/min crosshead speed of mechanical test machine (Instron 5565 A). Equipped with 0.5 kN load cell. The scaffolds were cut into suitable size of 10 mm wide and 60 mm length and a digital micrometer was used to measure the thickness of nanofibrous membrane. Force was applied to each specimen and stress-strain curve were recorded by inbuilt software.

^{1}H and ^{13}C NMR spectroscopy of nylon, nylon-*B. vulgaris*, and *B. vulgaris* solutions were performed in formic acid on a Bruker AVANCE 300 MHz nuclear magnetic resonance spectrometer with a TMS internal standard. Chemical shifts are reported in units of parts per million downfield from TMS.