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Biomimetic scaffolds containing nanofibers coated with willemite nanoparticles for improvement of stem cell osteogenesis

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ABSTRACT

Nowadays, discovering osteogenesis stimulating effectors is one of the major topics in bone tissue engineering and regenerative medicine. In this study, the proliferation rate and osteogenic differentiation potency of adipose-derived mesenchymal stem cells (AT-MSCs) cultured on poly (L-lactide acid) (PLLA) and willemite-coated PLLA were investigated by MTT assay and common osteogenic markers such as alkaline phosphatase (ALP) activity, calcium mineral deposition and bone-related genes expression.
Willemite-coated PLLA showed a higher proliferation support to AT-MSCs in comparison to PLLA and TCPS. During the period of study, AT-MSCs cultured on willemite-coated PLLA scaffolds exhibited the greatest ALP activity and mineralization. Gene expression analysis demonstrated that the highest expression of four important osteogenic-related genes, osteonectin, Runx2, collagen type 1 and osteocalcin was observed in stem cells cultured on willemite-coated PLLA nanofibrous scaffolds. According to the results, willemite-coated PLLA could be a suitable substrate to support the proliferation and osteogenic differentiation of stem cells and holds promising potential for bone tissue engineering and regenerative medicine applications.

1. Introduction

There exists an emerging need for introduction of novel strategies to regenerate and repair organ failure [29]. Tissue engineering holds promising potential as an alternative solution for tissue and organ failure treatment which is produced as a result of injury or other damage types [29]. Conventionally, when tissues or organs failure occurred, grafting natural, synthetic, or semi-synthetic tissue and organ mimics is considered. Such a tissue mimic may be totally operative from the beginning or might regain its original properties and functions as it grows [33].

In developed countries, the vast majority of chronic pains in people older than 50 years is the consequence of musculoskeletal conditions, in which patients suffer from drastic long-term pain and physical disorders [49].With respect to Bone and Joint Decade (2000–2010) which was officially announced by World Health Organization, it might be stated that the recent advancements in bone defect regeneration are the forefront area of tissue engineering [4,49]. Tissue engineering approaches for bone repair and regeneration are based on stimulating bone formation by using growth factors and/or cells in combination with a biomaterial scaffolds [34–36].

Recently, synthetic bone graft substitutes (BGS) usage is turning into one of the most important strategies for bone regeneration. BGSs are classified into several groups such as: polymers, ceramics and metals or composites made of these materials based on their material family, origin, physiochemical and structural properties [21,26]. BGSs are ideal when resemble extracellular matrix (ECM) structure, and can be osteoinductive [12] (i.e. they induce commitment of undifferentiated cells to become osteoblasts) and/or osteoconductive [20] (i.e. provide a framework for bone ingrowths). It is well established that both osteoinductivity and osteoconductivity can be seen in ceramic based BGSs especially in the form of calcium phosphate [26] (CaP). Moreover, according to some recently published reports, a few metals including porous titanium (Ti) and zinc also demonstrate these properties [3,13].

One of the major topics in the bone healing research area is to accomplish research on the osteogenesis stimulating effects of trace elements. Zinc is a crucial trace element which has variety of roles in human and numerous animals' growth such as increasing bone protein, calcium content, and alkaline phosphatase activity as well as bone metabolism [17,18,22,47]. Zinc deficiency is concomitant with bone growth slowness and defects, both in animals and humans [15,23]. Also, zinc has been shown to stimulate bone formation and osteoclast activity inhibition. The bioactive glass containing ZnO could enhance osteoblast activity and facilitate bone formation [5,25,28,38,51]. In 2002,

Abbreviations: PLLA, poly-L-lactic acid; BGS, bone graft substitutes; AT-MSC, adipose tissue mesenchymal stem cell; ALP, alkaline phosphatase; TCPS, tissue culture polystyrene.

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Ito et al. developed a zinc-containing b-tricalcium phosphate (bZnTCP) ceramic. Findings suggested that their new synthesized biomaterial promotes bone formation [17]. On the other hand, imitating the ECM is an efficient method for designation and development of appropriate scaffolds in regenerative medicine. Since the first bio-active glass introduction for bone tissue engineering silicon containing biomaterials attracted attention. Further examinations have also confirmed that bio-glass and silicate bioceramics promote higher osteoblastic gene and protein expression. Zhang et al. reported preparation and characterization of novel willemite nanoparticles which could potentially be used for osteogenesis [41,45,50].

We have recently developed various kinds of poly (L-lactic acid) (PLLA) based nanofibers and demonstrated their ability to enhance osteogenic differentiation of stem cells [8,19,34,35]. In this study, considering all the properties stated about Zn, silicates and PLLA based nanofibers, we coated willemite (Zn_2SiO_4) nanoparticles on PLLA nanoscaffolds. Afterwards, human adipose derived mesenchymal stem cells osteogenic differentiation potency is investigated on these nanoscaffolds.

2. Materials and methods

2.1. Scaffold fabrication

Electrospinning was employed for nanofibrous scaffolds production according to the protocol previously reported by our group [34]. In brief, PLLA was dissolved in dichloromethane (Merck, Germany) in order to make 12% (*w*/*v*) solution. The solution was placed in a 5 mL syringe. By a tube, the syringe was linked to a 21-gauge. A steel cylindrical collector was employed for electrospun nanofibers pileup 15 cm away from the needle. Pushed by a pump, the solution was sprayed very slowly (approximate rate was 1 mL/h). Using a 20 kV voltage in the electrospinning machine, the mentioned solution will be converted to fibers collected on the cylinder. The process is continued until the thickness of the mat become almost 200 μm, and then the mat is freed from the cylinder and remaining solvent dried in a vacuum.

2.2. Preparation of willemite ceramics

Willemite powders were produced according to the reported protocol by Zhang et al. [52]. SiO2 (99.9%) and ZnO (99.9%) were used as raw materials in solid reaction process. Briefly, ZnO and SiO2 powders were mixed with each other by ball milling in ethanol for 24 h. Next, the product was dried at 60 °C, ground and calcined at 1250 °C for 2 h. Using 6% Polyvinyl alcohol for binding the resulting powders were pressed uniaxially at pressure of 10 MPa to form rectangular compacts (45.5 mm ∗ 8.0 mm ∗ 3.5 mm). On the following step, cold isostatic pressing at 200 MPa was utilized. The green samples were subsequently formed at 1250, 1300, and 1350 °C for 2 h, respectively.

2.3. Surface modifications

Firstly, fabricated PLLA scaffolds were treated by oxygen plasma in a 44 GHz frequency plasma generator (Diener Electronics, Germany). Pure oxygen was put into the chamber at pressure of 0.4 mbar and the glow discharge was initiated about 5 min. Then, using ultrasonic bath for 20 min, willemite nanoparticles were dispersed in deionized water to make 1% (*w*/*v*) solution. In the next step, PLLA mats were buried in willemite suspension overnight to deposit nanoparticles on the surface of nanofibers. Finally, PLLA scaffolds were washed with deionized water and dried under the hood.

2.4. Stem cell isolation and expansion

For isolation of mesenchymal stem cell from adipose tissue (AT-MSC), adipose tissue obtained from cosmetic liposuction from ten different donors (age 18–40, Taleghani Hospital, Tehran, Iran) with their full accept and willingness according to Iran's Ministry of Health Medical Ethics Committee guidelines. Given tissues were treated with 0.2% collagenase II under intermittent shaking in an incubator about 30 min. Next, the samples were centrifuged, and the cell pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (*v*/*v*) fetal bovine serum (FBS) and cultured in flasks. When flasks became confluence, they were passaged and subcultured till passage number 5. For osteogenic differentiation, basal medium (DMEM supplemented with 10% FBS) was supplemented with 10^{-9} M dexamethasone (Sigma), 50 μg/mL ascorbic acid 2-phosphate (Sigma), and 3 mM beta-glycerophosphate (Sigma).

2.5. Cell seeding

Before cell seeding, the PLLA scaffolds were cut into 1.5 cm diameter circular shape to fit in 24 well plates (TCPS) and sterilized by immersion in 70% ethanol for about 2 h. After that, for facilitation of cell attachment, the scaffolds were submerged in DMEM overnight. An initial cell density of 2×10^5 cells per cm^2 were suspended in 200 μL of medium and seeded onto the PLLA, willemite-coated PLLA scaffolds and tissue culture polystyrene (TCPS) as control and incubated for 30 min. After doing so, 800 μL basal medium was added to each well. One day later, all the scaffolds were moved to new wells and treated with osteogenic medium for 21 days. The medium was replaced every two days.

2.6. Scanning electron microscopy (SEM)

Surface modification of the PLLA was characterized by scanning electron microscopy (SEM). Using a sputter coater, samples were coated with gold and studied by a microscope. The diameter of the fibers was calculated from SEM images by image analysis software (imageJ, NIH, U.S.A.). Morphology of cultured cells was also investigated in differentiation period. The cell-loaded scaffolds were rinsed with PBS after 7 and 14 days of osteogenic differentiation and fixed in 2.5% glutaraldehyde solution for 1 h. Then the scaffolds were placed in a series of gradients of alcohol concentration for dehydration and vacuum dried.

2.7. ATR-FTIR spectroscopy

Willemite coating was analyzed by FTIR spectroscopy. Equinox 55 spectrometer (Bruker Optics, Germany) was used to record the spectra.

2.8. X-ray diffraction

Willemite coated PLLA scaffold was analyzed by X-ray diffraction (Equinox 3000, INEL, France). The diffractometer was operated at 40 KV at a 2θ range between 10 and 90°.

2.9. Mechanical properties

The scaffold tensile strength was measured by Galdabini testing equipment. Scaffolds were cut into 10 mm × 60 mm shapes and tensile test was performed at 50 mm/min rate at room temperature.

2.10. Flow cytometry

After 10 days of AT-MSC isolation, expression of surface markers was evaluated by flow cytometry. AT-MSC were detached with trypsin treatment and incubated with either CD??, CD??, antibodies or isotope control antibodies in 200 μL 3% (*w*/*v*) bovine serum albumin in PBS for 1 h at 4 °C. The cells were then fixed in 1% PFA and analyzed with flow cytometer.