International Journal of Biological Macromolecules xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Poly(L-lactic acid) nanofibers containing *Cissus quadrangularis* induced osteogenic differentiation *in vitro*

Parvathi K, Amit G. Krishnan, Anitha A, R. Jayakumar*, Manitha B. Nair*

Center for Nanosciences and Molecular Medicine, Amrita Vishwa Vidyapeetham, Kochi, Kerala, 682041, India

ARTICLE INFO

Article history:
Received 18 September 2017
Received in revised form 9 November 2017
Accepted 14 November 2017
Available online xxx

Keywords:
Cissus quadrangularis
Electrospinning
Osteoinduction

ABSTRACT

Cissus quadrangularis (CQ) is known as "*bone setter*" in Ayurvedic Medicine because of its ability to promote fracture healing. Polymers incorporated with CQ at lower concentration have shown to enhance osteogenic differentiation of mesenchymal stem cells (MSCs) *in vitro*. However, for the healing of clinically relevant critical sized bone defects, large amount of CQ would be required. Based on this perception, a herbal fibrous sheet containing high weight percentage of CQ [20,40 and 60 wt/wt% in poly (L-lactic acid) (PLLA)] was fabricated through electrospinning. The solution concentration, flow rate, voltage and tip-target distance was optimized to obtain nanofibers. The hydrophobicity of PLLA fibers was reduced through CQ incorporation. There was considerable increase in the adhesion, proliferation and osteogenic differentiation of MSCs on herbal fibers than normal fibers, mainly on P-Q20 and P-CQ40. MSCs were differentiated into osteoblasts without providing any osteogenic supplements in the medium, indicating its osteoinductive capability. The herbal sheet also could promote mineralization when immersed in simulated body fluid for 14 days. These studies specify that PLLA nanofibers loaded with 20 and 40 wt% of CQ could serve as a potential candidate for bone tissue engineering applications.

1. Introduction

An ideal bone tissue engineered scaffold should mimic the native extracellular matrix (ECM), consisting of collagen nanofibers and nano-hydroxyapatite. Bone grafts can be prepared by several methods and among which, electrospinning is a simple and cost-effective technique that develops micro or nano fibers [1]. The technique also helps to encapsulate, and release drugs over a long period of time in sustained manner [2]. However, tissue regeneration or drug release kinetics depends on the nature of the polymer used for electrospinning. Poly(L-Lactic acid) has been widely used as the material of choice in tissue engineering and drug delivery applications owing to its appropriate mechanical and biodegradable properties [3]. However, PLLA is hydrophobic and lacks cell adhesion moieties. In addition, electrospun PLLA is not osteoinductive in nature, limiting its bone regeneration potential [4]. In order to improve the hydrophilicity as well as osteoinductive properties, growth factors were incorporated into electrospun PLLA fibers, which trigger the migration or proliferation of stem/progenitor cells by providing physical and biochemical environment. Nonetheless, growth factor mediated treatment strategy is really expensive [5]. This necessitates cost effective approaches to enhance the biological properties of synthetic polymeric scaffolds.

Cissus quadrangularis is used in Ayurvedic medicine for centuries and is known as "bone setter" for its ability to join bones [6,7]. It is also shown to be anti-osteoporotic, anti-microbial and anti-inflammatory etc. This therapeutic drug comes in the form of capsule, tablet and syrup and the daily adult dose is around 500–1000 mg [6]. The effects of CQ on the proliferation and osteogenic differentiation of bone marrow derived MSCs have been well studied [8]. It is demonstrated that CQ paste can decrease the healing time of fractures induced in the radius and ulna of dogs [9]. In a clinical study, CQ has shown to accelerate the regeneration of maxillofacial fracture [10]. The administration of 500 mg/kg of CQ in an ovariectomized osteoporotic rat model for 3 months resulted in an increase in alkaline phosphatase (ALP) activity and new bone formation [11]. The capability of CQ to prevent bone loss was also shown in an ovariectomized mice model [12].

Although the osteogenic effect of CQ has been well documented, there are only few reports showing the fabrication of scaffolds using this drug. A herbal hydrogel of alginate-carboxymethyl chitosan and CQ was developed by our group in 2012 [13]. In another report, CQ extract was integrated into sulphonated poly(aryl ether ketone) sponges through lyophilisation technique [14]. There is only one

* Corresponding authors.
E-mail addresses: rjayakumar@aims.amrita.edu (R. Jayakumar), manithanair@aims.amrita.edu (M.B. Nair).

https://doi.org/10.1016/j.ijbiomac.2017.11.094
0141-8130/

study demonstrating the creation of nanofibrous scaffolds with CQ (polycaprolactone (PCL)-CQ-HA) by electrospinning technique [15]. In all these studies, CQ drug was incorporated into the polymer at lower concentration and has resulted in beneficial effects *in vitro*. However, for the healing of critical sized segmental bone defects that occur due to trauma or osteosarcoma, large amount of CQ would be preferred at the defect.

In this study, we report for the first time, a herbal fibrous sheet by blending high weight percentages of CQ extract (20, 40 and 60 wt/wt%) with PLLA. The solution concentration, flow rate, voltage and tip-target distance was optimized to obtain nanofibers. The capability of herbal biomaterial to induce osteogenic differentiation was verified *in vitro*.

2. Materials and methods

2.1. Materials

PLLA granules were purchased from Good Fellow Cambridge, England. Ethyl alcohol (Analytical reagent, 99.9% purity) was obtained from the Institute after obtaining consent from the Excise department of Govt. of Kerala, India. Chloroform, methanol (HPLC grade), CHAPS, sodium dodecyl sulphate, bicinchoninic acid (BCA) kit, ALP enzyme, p-nitrophenyl phosphate and gluteraldehyde were obtained from Sigma Aldrich; α-minimal essential medium (α-MEM), fetal bovine serum (FBS) and antibiotic-antimycotic solution were purchased from Gibco, India. Alizarin Red Stain was from Nice chemicals, India. Lactate dehydrogenase (LDH) from Promega, India and collagenase type I enzyme was from Invitrogen, USA.

2.2. Extraction of Cissus quadrangularis

Cissus quandrangularis was collected from Tripunithura Ayurveda College premises and authenticated by the quality assurance department of Arya Vaidya Sala Kottakkal, Kerala, India. The plant was collected from one source and used for the entire studies in order to avoid batch to batch variation. The stem of CQ was washed thoroughly with double distilled water; shade dried; cut into small pieces; powdered in a pulvarizer and extracted in a Soxhlet apparatus with 99% ethanol at 60° C. The obtained extract was further distilled in an apparatus to remove the excess alcohol content and stored in a sterile container at −20 °C until use. The yield of CQ extract was 5–6 g, which was obtained from 50 g of dried plant.

2.3. Fabrication of herbal fibrous sheet

The solution concentration, voltage, spinneret-collector distance was optimized to obtain PLLA nanofibers (Table 1). In short, herbal fibrous sheet was prepared by dissolving CQ extract (20, 40 and 60 wt/wt) in PLLA solution (7 and 9 wt% PLLA in 1:3 chloroform: methanol solvent). The solution was stirred for 2 h and loaded onto 10 mL syringe with blunt ended metal needles attached to a customed electrospinning set up that consist of high-voltage DC power supply (Model RR30P, 0–30 kV, Gamma High Voltage Inc., USA), syringe pump (KDS 220, KD Scientific Inc., USA) and a motorised translational target. The sheet was spun at a flow rate of 0.8 and 1 mL/h with an applied voltage of 10, 15, 18 and 20 kV and tip to target distance of 10 and 13 cm. Random fibers were collected on a collector plate wrapped with aluminium foil. Four groups were included in the study, which includes (a) PLLA (b) P-CQ20 (c) P-CQ40 (d) P-CQ60.

2.4. Characterization of herbal fibrous sheet

The surface morphology of electrospun sheets was studied by scanning electron microscope (SEM) (JEOLJSM-6490LA) at an accelerating voltage of 15 kV, after sputter coating with gold (JEOL JFC-1200fine coater, Japan). The diameter of the electrospun fibers were analyzed from the SEM images using image analysis software (Image J, National Institutes of Health, USA). FTIR spectroscopic analysis of electrospun sheets was performed (Avatar 380, Thermo Nicolet, Waltham, MA) over a range of 400–4000 cm^{-1}. Hydrophilic nature of the electrospun sheets was measured by sessile drop water contact angle measurement using VCA Optima Surface Analysis system (AST products, Billerica, MA). Tensile properties of electrospun sheets (5 × 1 cm) were determined with a tabletop tensile tester (Instron3345, USA) using 10 N load cell.

2.5. Protein adsorption

Quantitative evaluation of proteins adsorbed onto the surface of the electrospun sheet (1 cm x 1 cm) was measured using BCA assay (n = 6). For this, PLLA (normal and herbal sheets) were incubated in 500 μL FBS for 2 and 6 h at 37° C, washed with phosphate buffered saline (PBS) and the adsorbed proteins were eluted using an elution buffer made up of 0.5% CHAPS and 10 wt% sodium dodecyl sulfate. The total amount of protein adsorbed by the scaffolds was measured spectrophotometrically using Biotek (Power Wave XS, USA) at a wavelength of 562 nm.

2.6. Cytocompatibility assay

Rat MSCs were isolated from adipose tissue after getting approval from the Institutional Animal Ethics Committee. Briefly, the harvested tissue was minced; digested in 0.1% collagenase type I enzyme for 20 min at 37 °C and centrifuged at 1500 rpm for 10 min. The pellet was suspended in α-MEM containing 20% FBS, antibiotic-antimycotic solution; plated onto T25 tissue culture flasks and incubated at 37 °C in a humidified atmosphere at 5% CO_2. Media change was given for every two days. After attaining 80–90% confluency, the isolated cells were characterized by CD105, CD90 and CD44, as previously reported [16]. MSCs at passage 2–3 (5×10^4 cells) were seeded onto the sheets (PLLA, P-CQ20, P-CQ40, P-CQ60 with a dimension of 1 cm × 1 cm) and incubated for day 1, 7 and 14 in basal medium without providing any osteogenic supplements.

2.6.1. Cell morphology

The morphology of the cells grown on PLLA (normal and herbal sheets) was observed after 24 h by SEM (n = 2). For this, the scaffolds were fixed in 1% gluteraldehyde for 24 h, dehydrated with increasing concentrations of ethanol (50, 70 and 80% for 10 min twice and 90 and 100% for 15 min each). The samples were coated with gold and visualized under SEM at an accelerating voltage of 15 kV.

2.6.2. Cell viability

The viability of MSCs on PLLA (normal and herbal sheets) was quantitated using LDH assay (n = 6). The cells on the scaffolds were lysed with 1% Triton-X-100 for 50 min including sonication for 10 min; the lysate was added to 50 μL LDH substrate; the enzymatic reaction was stopped after 30 min with 0.1 M acetic acid and the absorbance was read at 492 nm with a microplate reader (Biotek PowerWave XS). Finally, the cell viability was calculated using a calibration line constructed with known concentration of cells after 8 h of cell seeding.

2.6.3. Osteogenic differentiation

The osteogenic differentiation was assessed by ALP activity after 1, 7 and 14 days, based on the hydrolysis of p-nitrophenyl phos-