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Impact of pre-incubation time of silk fibroin scaffolds in culture medium on cell proliferation and attachment

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ABSTRACT

Cell behaviours such as proliferation and attachment can be affected by the length of pre-incubation period of the scaffolds in the culture medium for long term. The aim of this study was to investigate the long term pre-incubation of 3D silk fibroin scaffolds in complete culture medium on cell attachment and proliferation. After the preparation of silk fibroin scaffolds by the technique of freeze drying, the scaffolds were pre-incubated in complete culture medium for 2 d, 6 d or 10 d before apical papilla stem cells (SCAP) seeding. Modifications of the scaffold surface and wettability were examined by FE-SEM and water contact angle, respectively. Results showed a decrease both in roughness and water contact angle as pre-incubation time increases. DNA measurement after 18 h and 10 d cell seeding showed a significant increase of DNA concentration which represents better attachment and proliferation with pre-incubation time increase. Qualitative examination, live & dead assay or H & E staining method after 30 h and 10 d cell seeding respectively, indicated that pre-incubation of scaffolds has time dependent effect on cell proliferation and attachment. This suggests that improvement of cell attachment and proliferation may be mediated by differences in the amount of wettability (decreased water contact angle) after exposure of scaffold to culture medium for long term which, in turn, causes more protein adsorption in the surface of silk fibroin scaffold (decreased roughness).

1. Introduction

The aims of tissue engineering as a promising technology is to repair damaged organ or tissues using cells and biomaterials (Griffith and Naughton, 2002; Zhang et al., 2010). The cells cannot make direct contact when they encounter with such biomaterials but these constructs adsorb proteins from serum or blood that make them biocompatible (Leavesley and Pearcy, 2005). Similar to a natural environment in which cells anchor to the protein in extracellular matrix (ECM), cell attachment in vitro to culture surfaces also occurs through adhesion proteins existed in complete culture medium (Hayman et al., 1985; Leavesley and Pearcy, 2005; Schneider and Burridge, 1994). Proteins contained in serum will attach to polymer competitively for they have different surface affinities (Fabrizius-Homan and Cooper, 1991; Slack and Horbertt, 1991). Over time (hours), adsorbed layer containing proteins with faster diffusing property (e.g., albumin) is replaced by one that has heavier molecular weight but higher affinity. This is often defined as the Vroman effect (Jung et al., 2003; Noh and Vogler, 2007; Wertz and Santore, 1999).

Protein adsorption and its effects on 3D scaffolds were also investigated. Fabrication of 3D polymer scaffolds using precision extrusion deposition technique and soaking in fibronectin solution showed significant increases in cell adhesion (Yildirim et al., 2010). Treatment of composite scaffolds made of poly (L-lactic acid)/hydroxyapatite (PLLA/HAP) with FBS and then with fibronectin and vitronectin significantly reduced apoptosis (Woo et al., 2007). However, few in vitro studies have been performed to identify the long term effects (days) of 3D scaffold pre-incubation in aqueous and physiological medium on cell behaviours. Polymer properties (degradation, swelling, surface coating, modulus, hydrophilicity) will change by incubation in such mediums. All of these changes may result from polymer rearrangement in scaffold surface (Elliott et al., 2003; Ratner et al., 1978) that may become time-dependent and severe with increasing length of polymer incubation in physiological medium. The poly (D,L-lactic acid) (PDLLA) film pre-aged in medium with serum had enhanced cell adhesion and spreading (Chen et al., 2008). In addition, pre-aging of Poly (Ɛ-caprolactone) (PCL) 3D scaffold fabricated by porogen-leaching up to 7 days in culture medium improved cell proliferation but not attachment

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(Chatterjee et al., 2012). One of the events that occur in pre-incubation of biomaterials in physiological medium is the change of wettability over time that may affect cell attachment and proliferation.

To test this hypothesis we performed the pre-incubation of sponge silk fibroin scaffold fabricated by the freeze-drying method in complete culture medium and then we studied its effects on proliferation and adhesion of stem cell of the apical papilla (SCAP) via DNA measurement and cell staining. Water contact angle and FE-SEM were used immediately after incubation to examine the hydrophilic properties and qualitative surface modification of the scaffold, respectively. The biocompatibility, mechanical strength and the slow degradation rate suggest that silk has a potential role in bone repair (Andiappan et al., 2013; Kuboyama et al., 2013; Mandal and Kundu, 2009; Zhang et al., 2010). SCAP represent an early stem/progenitor cells which proliferate 2–3 fold greater than other sources and have a good potential to osteoblast differentiation, therefore, they will be a superior candidate for bone tissue regeneration (Huang et al., 2009; Huang et al., 2008; Ponnaiyan, 2014; Sonoyama et al., 2008).

2. Material and method

2.1. Materials

Cellulose dialysis tube with cut off 12,400 Da (Sigma, USA), polystyrene plastic flasks and plates with tissue culture grade (SPL, Korea), Dulbecco's modified eagle medium (DMEM, Gibco, USA), FBS (Gibco, USA), trypsin–EDTA (Gibco, USA), penicillin–streptomycin antibiotics (Gibco, USA), collagenase type I (Sigma, USA), Live & Dead assay (Abcam, UK). All other reagents were of analytical grade.

2.2. Isolation of silk fibroin protein from cocoons

Silk fibroin protein was isolated from mulberry silk cocoons by a standard procedure of isolation (Sofia et al., 2001). Briefly, the cocoons were minced (~1 × 1 cm) and the pieces were degummed by boiling them in 0.02 M Na_2CO_3 (10% w/v of cocoon pieces and aqueous solution of Na_2CO_3) for 30 min. After washing the degummed silk fibers for several times in distilled water, they were dried in room temperature overnight. Dried silk fibers were then dissolved in 9.3 M lithium bromide for 5 h at 55 °C and subsequently obtained solution was dialyzed against deionised water for 3 days to remove salt lithium bromide. Frequent changing of dialysis water was performed as well. Finally to remove insoluble materials, the obtained solution was centrifuged two times at 9000 RPM for 20 min each time at 4 °C (Rockwood et al., 2011).

2.3. Fabrication of sponge silk fibroin scaffold

Freeze-drying method was used to prepare sponge 3D silk fibroin scaffolds. After cooling the falcon tubes to 4 °C, 5 wt.% of silk fibroin solution was poured into them, and then to solidify the silk fibroin solution, the falcon tubes were immediately transferred to a freezer (–80 °C) for 12 h. The solidified mixture was freeze-dried (CHRIST Alpha 1–2, Germany) for 36 h until completely dry. Then crystallization of the fabricated scaffold was induced by treating the samples with methanol for 1 h.

2.4. Porosity of scaffolds

Liquid displacement method was performed to measure the porosity of the silk scaffolds (Kim et al., 2005; Zhang and Ma, 1999). Hexane was used as a displacement liquid in this test because it can simply permeate into the scaffolds but does not cause swelling or shrinking of the silk fibroin scaffolds. Known volume of hexane (V_1) was poured into a measuring cylinder and then silk scaffold was immersed in it for 10 min. After immersion, total volume of hexane and the scaffold was considered as V_2. Then V_3 was considered for remaining volume in the measuring cylinder after removing hexane-impregnated scaffold. The porosity was calculated using the following formula:

$$\text{Porosity\%} = (V_1 - V_3)/(V_2 - V_3) \times 100.$$

2.5. Water uptake

The sponge silk scaffold was placed into distilled water for 24 h at room temperature. After removing excess water, wet scaffold was weighed and recorded (W_s). Next, the wettability of scaffold was evaporated by placing into oven for 24 h at 50 °C. The weight of dried scaffolds was then recorded as W_d (Venkatesan et al., 2014). The percentage of water uptake was calculated with the following formula;

$$\text{Water uptake (\%)} = [(W_s - W_d)/W_s] \times 100$$

2.6. Scanning electron microscope

The sponge silk fibroin scaffolds were coated with gold particles before pre-incubation to study the scaffold morphology. Gold coated specimens were imaged by SEM (Philips, Netherland). Pore sizes of scaffold were determined by measuring random 30 pores from SEM micrograph using Image J software.

2.7. XRD

X-ray diffraction (XRD) of silk fibroin scaffold was examined with radiation of Cu-Ka (λ = 0.15405 nm) using Philips X'Pert 1 X-ray diffractometer. The measurement range was 2θ = 5–35°.

2.8. FTIR spectroscopy

Freeze-dried silk fibroin scaffold was powdered and the 1 mg of powder was combined with 200 mg of potassium bromide to press into a pellet. Fourier transform infrared (FTIR) spectra in the spectral region of 500–2000 cm^{-1} were recorded using a Galaxy Series FTIR 5000 system.

2.9. Isolation, characterization and expansion of SCAPs

Third molar apical papilla tissues were obtained from normal human impacted teeth (18 years of age) with informed consent and under the Dental Clinic guidelines approved by the Ethics research Committee of Babol University of Medical Sciences. From the root, apical papilla tissue was gently separated and cut into small pieces. For digestion, apical papilla pieces were placed into the collagenase type I solution (5 mg/ml) for 1 h at 37 °C. Released single cells were harvested by centrifuging and the pellet was suspended and cultured in DMEM containing 15% FBS, 100 mg/ml streptomycin and 100 U/ml penicillin at 37 °C in 5% CO_2 (Sonoyama et al., 2006; Yang et al., 2012).

The presence of positive (CD105,CD90 and CD 73) and negative (CD45 and CD34) specific mesenchymal stem cell antigens in the surface of apical papilla stem cells (passage 3) were evaluated using flow cytometry (Sonoyama et al., 2006). In addition, in vitro differentiation potential of cells into the adipogenic and osteogenic lineages was evaluated by the induction of adipogenic and osteogenic medium up to 21 days and then followed by the analysis of mineralization and formation of neutral lipid using the Alizarin Red and Oil Red O staining, respectively (Soleimani and Nadri, 2009).

2.10. Pre-incubation and Cell Seeding

Samples were cut into cylinder shapes (6 mm in diameter, 3 mm in height). The cylinder-shaped scaffolds were then transferred into dishes