CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.1914

The Suitability of Silk Fibroin Based Biofilms for Cartilage Tissue Engineering

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Cite this article as: Nwekwo CW, Kalkan R, Adali T. The Suitability of Silk Fibroin Based Biofilms for Cartilage Tissue Engineering. Cyprus J Med Sci 2021; 6(2): 124-128.

BACKGROUND/AIMS
Silkworms and spiders produce silk fibroin (SF). SF protein has unique characteristics, which includes mechanical properties, biodegradation, biocompatibility, and the ability to support the differentiation of stem cells along the osteogenic lineage. These characteristics makes SF a favorable biomaterial for cartilage tissue engineering. The aim of this study was to design a SF biofilm and then to test the biocompatibility and cytotoxicity of the designed SF biofilm.

MATERIAL and METHODS
Characterization was executed by scanning electron microscopy and Fourier transform infrared spectrophotometer analysis spectrophotometry. Normal human articular chondrocytes were seeded on biofilm and cultured up to 2 weeks (5% CO_2, 95% air and 37°C) under the standard culture conditions. Phase contrast microscopy and cell proliferation assay (3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay) was applied for evaluation of cell attachment and cell growth.

RESULTS
The viability of cells was linearly correlated with optical density, and chondrocyte viability in the SF film was found to be significantly higher.

CONCLUSION
These results indicated that SF film supports cell proliferation without side effects and the SF film is a potential material as a cartilage tissue engineering matrix.

Keywords: Biofilm, silk fibroin, chondrocyte, MTT, tissue engineering

INTRODUCTION

Adult articular cartilage has a limited self-repair capacity.[1] Repair and/or regeneration of articular cartilage defects is challenging in orthopedics.[2] Autologous cell-based tissue engineering holds a promising option for the repair trauma or aging related cartilage damages.[1] Cell based therapies or tissue engineering applications for in vitro cartilage tissue development need cells that are able to undergo chondrogenic differentiation with appropriate growth factors and suitable biomaterials, which provides a favorable microenvironment of cell growth and new cartilage-specific extracellular matrix (ECM) formation.[1]

There are different types of biomaterials that can be used for cartilage repair such as naturally occurring, synthetic, biodegradable, and nonbiodegradable materials.[3,4] Silk fibroin (SF), chitosan (CHI), hyaluronan, and alginate are most frequently used polymers in cartilage tissue engineering for development of ECM in tissue regeneration applications.[5–8]

Silk proteins are produced from cocooned silk worms or spiders.[9–11] High biocompatibility capacity,[12] robust mechanical properties, biological compatibility, and blood compatibility make SF protein an important natural biomaterial in tissue engineering.[13]

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Received: 27.03.2020
Accepted: 12.11.2020

In the present study, characterization of films was carried out for cartilage tissue engineering application. Swelling kinetics, biodegradation, and antimicrobial activity of the biofilms were assessed. Then, designed SF based biofilms were used for the evaluation of cell proliferation activity of Normal Human Articular Chondrocyte (knee, NKAC-kn) cells.

MATERIAL and METHODS

Mulberry *Bombyx mori*, cocoons were collected during the harvesting time from Lapta, North Cyprus.

Preparation of SF Film

The processes involved in the synthesis of the pure SF were as follows:

- degumming process,
- dissolution process, and
- dialysis process.

In the degumming process, cocoons were cut into small pieces, and 1 g of the cocoon was weighed and put into a conical flask. Then, 100 mL of the 0.1 M sodium carbonate Na_2CO_3 solution (1%, w/v) was added into the same conical flask. Degumming process was carried out on magnetic stirrer at 1 rpm and 70°C for 3 hours. The process was repeated to ensure that sericin, the gum, was entirely removed from the silk cocoon and SF protein was dried at 25°C.

Dissolution Process

The dissolution process changes the physical state of the SF from solid to liquid and the chemical state by breaking the H-bonding of SF protein. The strong electrolyte solution with molar ratio: n_{C2H5OH}:n_{CaCl2}:n_{H2O}; 2:1:8. The required amount of degummed SF fibers were added into strong electrolyte solution and stirred at 1 rpm at 75°C. This was continued until the SF was totally dissolved.

Dialysis Process

The dialysis process was used to purify liquid SF protein. A semipermeable SnakeSkin® Dialysis Membrane (10,000 MWCO) was used for dialysis process. The pure SF was removed from the membrane using a syringe.

Preparation of the Silk Fibroin Biofilm

Five grams of the pure SF was then measured, placed into a 25 mL beaker, and eft to dry overnight at 25°C. Methanol was added to cause beta-sheet formation and later removed. The biofilm is rinsed and left to dry at room temperature.

Characterization of SF Film

Fourier transform infrared spectrophotometer analysis (in Cyprus International University, Cyprus) and scanning electron microscope analyses (Jsm-6510 model at an acceleration voltage 10 kV, at Middle East Technical University, Turkey) were carried out in order to characterize the SF biofilm produced. The SEM device produced images of the samples by focusing beam of electrons on it, and samples were coated with gold to prevent charging.[14]

Main Points

- SF film supports cell proliferation without side effects.
- The SF film is a potential material as a cartilage tissue engineering matrix.
- The SF is a potentially function as a promising articular cartilage substitute for tissue engineering applications.

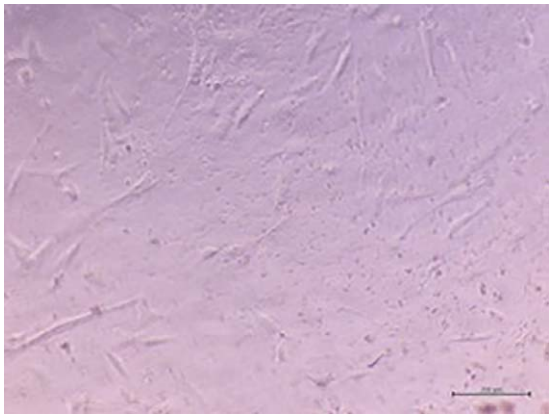

Figure 1. Cell attachment of NHAC-kn cells with phase contrast microscope; 7 days after seeding on biofilm

Antibacterial Activity

Kirby-Bauer disk diffusion method was used for the evaluation of antimicrobial activity of SF biofilm.[15] It was carried out with three gram-negative bacteria and three gram-positive bacteria. The zone of inhibition of each biofilm was measured using a scale, and photographs of the different petri plates were taken (data not supplied).

Cell Culture

NHAC-kn (Lonza, Clonetics™ Normal Human Articular Chondrocyte Cell System) culture was prepared as described previously,[14] and phase contrast microscope Olympus IX53 with camera Olympus DP22 was used for identification of cell morphology.

SF biofilms were incubated in 24-well plates in culture medium for 4 hours at 37°C before cell seeding as described previously.[14] The chondrocytes were seeded on to top of the SF biofilm at a total density of approximately 1.25×10^4 cells/ml. and growth medium replenished after 24 hours. SF biofilms were incubated for 15 days with media changes every other day. Cell attachment (30 minutes, 1, 2, and 3 hours) was evaluated by using a phase contrast microscope (Olympus IX53 with camera Olympus DP22). All images were taken at 10× and 40× magnification (Figure 1).

Analysis of Cytotoxicity

Cellular metabolic activity and relative cell viability were analyzed by methyl thiazolyl tetrazolium (MTT, Invitrogen) assay. MTT assay was applied for evaluation of cell viability after the NHAC-kn cells were cultured in a 24-well plate for 7, 9, and 15 days. The MTT assay was performed as described previously[14] (Figure 2).

RESULTS

Cell Culture and MTT Assay

Phase contrast microscope micrographs of NHAC-kn seeded biofilm were taken after 24 hours, 48 hours, 7 days, 9 days, and 15

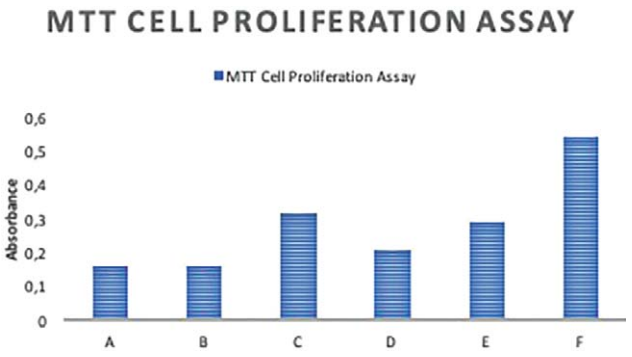

Figure 2. Cell viability in SF biofilm after 7 days, 9 days, and 15 days under the standard culture conditions. (A) Media only (0.157); (B) biofilm+media (0.157); (C) control NHAC-kn cell (0.313); (D) 7 days biofilm+cell (0.206); (E) 9 days biofilm+cell (0.289); (F) 15 days biofilm+cell (0.537).

days of culturing (data not shown). Cell growth was evaluated by measuring the MTT assay as shown in Figure 2. The viability of cells was linearly correlated with optical density; the chondrocyte viability seeded onto the SF based biofilm was significantly higher than controls. The chondrocyte viability on the SF biofilm was observed to significantly increase after 9 days.

DISCUSSION

SF is a nonsynthetic biomaterial used in tissue engineering applications. Various researchers used SF based films, fibers, meshes, membranes, scaffolds, hydrogels together with different natural materials like curcumin, hyaluronic acid (HA), poly(L-lactic-acid), CHI, cellulose, gelatin, CHI and hydroxyapatite, HA and fibrin/HA to test the cell proliferation activity, cell adhesion, and differentiation on these materials. Cartilage has a limited capacity for regeneration. Repair of cartilage injury by using specific cells, growth factors, and biomaterials is the main aim of cartilage tissue engineering. Selection of the biomaterial plays an important role within the process of cartilage engineering. Ideal scaffold should be biocompatible, biodegradable and have a three dimensional (3D) nanoporous structure.

Kim and colleagues worked with rabbit chondrocytes and used SF scaffolds and silk/curcumin blend scaffolds. They detected that curcumin with SF exhibited a high ECM production and provided an acceptable level of cell viability.[16] Nematollahi et al.[17] used rabbit chondrocytes together with silk-CHI blended scaffolds with glutaraldehyde (GA) concentrations. They concluded that tensile strength was raised with increase in freezing rate and GA concentration.[17] Zhang et al.[18] worked with bone marrow mesenchymal stem cells (BMSCs) on different materials such as collagen hydrogel (CH), collagen/sodium alginate hydrogel, collagen sponge, and silk sponge. They concluded that the induced and cocultured scaffolds exhibited high differentiation potential than control group.[18] Sawatjui et al.[19] used SF and SF-GCH scaffolds (SF/gelatine chondroitin sulfate (CS) hyaluron) with BM-MSCs and concluded that BM-MSCs proliferation, chondrogenic differentiation, and mimicking of cartilage structure and environment were identified in the SF-GCH scaffold.

Chomchalao et al.[20] used articular chondrocytes on SF, SF/C (SF/ceramic), and SF/G (SF/gelatin) scaffolds and enhanced cell attachment, proliferation of chondrocytes was observed on SF/C and SF/G scaffolds. Agrawal et al.[21] cultured hMSCs on SF:CHI (SF:CS) scaffold, and they detected cell distribution, proliferation, higher cell density, and increased expression of cartilage-specific genes. Li et al.[22] designed SF/CHI film and tried to identify rat bone marrow-derived mesenchymal stem cell proliferation activity on this material. They conclude that the SF/CHI based biofilm is a promising material for tissue engineering of bone, cartilage, adipose, and skin.[22] Wang et al.[1] used SF scaffolds and human articular chondrocytes successfully in cartilage tissue engineering. For bone tissue applications, contribution of the soluble form of ECM[23] to the SF improved wettability, mechanical property, and cyto-compatibility of the product by using a crosslinking method.[24,25] Zhou et al.[26] noted that together with SF, CS is safe and showed high biocompatibility for tissue engineering scaffolds. However, the problem is rapid degradation rate of pure CS scaffolds. Thus, SF is generally used because of its mechanical properties, long-lasting in vivo stability, and hypoimmunity.[26] They showed promoted articular cartilage defect repair with a combination of SF and CS and concluded that the silk-CS scaffold preserves better chondrocyte phenotype than silk scaffold.[26]

Lee et al.[25] developed SF (SF) and polyvinyl alcohol (PVA) hydrogels and used different methods, like salt leaching, silicone mold casting, and freeze–thawing methods, to test the auricular cartilage viability. They analyzed the swelling ratio, tensile strength, pore size, thermal properties, morphologies, and chemical properties of the hydrogels. These researchers found that the hydrogel, which is composed of 50% PVA and 50% SF (P50/S50), is the best-fabricated hydrogel for articular cartilage.[25] Shi et al.[27] used SF and gelatin scaffold to be able to repair cartilage injury via 3D printing technology. They detected high performance for cartilage repair in a knee joint and highlighted that the SF and gelatin combination scaffold was promising biomaterial for knee cartilage repair. Kim et al.[16] developed curcumin and SF scaffold for testing clinical replacement for defected cartilage. They detected cell proliferation and glycosaminoglycans (GAGs) and showed higher cell viability rate on 1 mg mL^{-1} curcumin/silk scaffold. Singh et al.[28] designed agarose/SF hydrogels of mulberry and nonmulberry SF. Higher amount of GAG and upregulation of cartilage-specific aggrecan, sox-9, and collagen type II was detected in nonmulberry SF hydrogel. They highlighted that the nonmulberry SF/agarose was an alternative biomaterial for cartilage tissue engineering.[28]

Jaipaew et al.[29] designed scaffolds from SF/HA and tested cartilage cell viability of this material by using human umbilical cord-derived mesenchymal stem cells (HUMSCs). They detected chondrogenesis related marker expression like Col2a, Agg, and Sox9 and suggested that the SF/HA scaffolds were suitable in cartilage tissue engineering and in surgery for osteoarthritis.[29] Ribeiro et al.[30] used horseradish peroxidase (HRP)-crosslinked SF scaffolds and cultured human adipose-derived stem cells. They detected under the chondrogenic culture conditions that cells showed adhesion, proliferation, and high GAGs synthesis and produced cartilage-specific extracellular matrix. They showed that the structural, mechanical, and biological performance of the proposed scaffolds could be used as 3D matrices for cartilage regeneration.[30]

Cai et al.[31] used SF coating PLA film systems for attachment and proliferation of rat osteoblasts. The usage of SF biomaterials on cartilage tissue regeneration has been used by many researchers.[1,32–36]

Tigli et al.[37] evaluated chondrogenesis by using different SF- or CS-based biomaterials and concluded that adoption of the cells was an important factor to be able to consider chondrogenic outcomes.[37] Silva et al.[38] used SF/CS composite scaffolds.

Proliferation of chondrocyte-like cells was observed in SF/CS composite scaffolds.[38,39]

SF based biomaterials, a combination of SF and other natural polymers, have been investigated for different cell types, like hMSCs, HUMSCs, and cartilage tissues. Due to its mechanical strength, elasticity, biocompatibility, bioactivity, adaptability, and biodegradability, SF protein has been widely used as a natural polymer in TE. Our results showed that, the NHAC-kn cell viability in the SF biofilm was significantly higher and indicated that SF film enhanced cell proliferation without side effects, and the SF film was a potential material for a cartilage tissue engineering matrix.

Natural polymers offer various advantages in cartilage tissue engineering applications but until now, there is no report to show ideal method for engineering of articular cartilage. SF is a naturally occurring protein, which has unique properties and that makes it a favorable matrix for the incorporation and delivery of a wide range of agents. This study highlighted that pure SF biofilm is an important material for tissue engineering applications for cartilage tissue engineering.

Ethics Committee Approval: N/A

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - R.K., T.A.; Design - R.K., T.A.; Supervision - R.K., T.A.; Resource - T.A.; Materials - R.K., T.A., C.W.N.; Data Collection and/or Processing - R.K., T.A., C.W.N.; Analysis and/or Interpretation - R.K., T.A., C.W.N.; Literature Search - R.K.; Writing - R.K; Critical Reviews - R.K., T.A., C.W.N.

Conflict of Interest: Authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Acknowledgments: The Near East University Scientific Research Project Unit supported this work [Grant Number: SAG-2016-02-017].

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