Original Article

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The effect of silk fibroin hydrogel on proliferation of human stem cells from the apical papilla

San Sakunphanitphan[1], Jeeeraphat Jantarat[2], Hathaitip Sritanaudomchai[3], Bovornlak Oonkhanond[4], Kenneth M Hargreaves[5]

[1] *Department of Operative Dentistry and Endodontics, Faculty of Dentistry, Mahidol University*
[2] *Department of Operative Dentistry and Endodontics, Faculty of Dentistry, Mahidol University*
[3] *Department of Oral Biology, Faculty of Dentistry, Mahidol University*
[4] *Faculty of Engineering, Mahidol University*
[5] *Department of Endodontics, University of Texas Health Science Center at San Antonio*

Objectives: The aim of this study was to compare the efficiency of various concentrations of silk fibroin hydrogel as a scaffold Proliferation of Human Stem Cells from the Apical Papilla (SCAPs).

Materials and Methods: Silk fibroin hydrogel (SF) was prepared at the concentration of 1.5% w/v, 2.0% w/v, and 2.5% w/v. An individual SF was seeded with SCAPs at 50,000 cells. Subsequently, MTT assay was used to analyse cell proliferation after 1, 7, 14, and 21 days of culture. Finally, the morphological features of SCAPs cultured on SF were investigated by live/dead assay. The Tukcy HSD followed by Dunn test was preformed ($P<0.05$).

Results: On SF at concentration 1.5%, SCAPs proliferation rate was highest compared to SF at concentration 2.0% and 2.5% significantly ($P<0.05$). Moreover, SCAPs on 1.5% SF exhibited more extension of cytoplasmic process and interconnected with neighboring cells than other SF concentration.

Conclusion: The findings from the current study suggest that 1.5% SF had a favorable effect on SCAPs proliferation. Further studies are required to investigated cell differentiation and the effect of microenvironment (in vivo) on cell and scaffold behavior.

Keywords: tissue engineering, scaffold, silk fibroin, stemcell(s), stem cell(s) of apical papilla

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Introduction

Principle of Regenerative Endodontic Procedures (REPs) is basically applied from regenerative medicine. Hargreaves et al., postulated 3 important components for the success of the procedure [1]. These include stem cells, growth factors or signaling molecules and three-dimentionals scaffolds that provide suitable environment for cell growth and differentiation. In clinical procedure, intracanal blood clot serves not only as a source of stem cells and growth factors, but also as autologous scaffold [2,3]. However, to create a blood clot this step is not always successful in every case [4,6]. Since this problem has been recognized, researchers have begun examining for the new three-dimensional scaffold that can be substitute of blood clot.

Silkworm silk from *Bombyx mori (B. mori)* composes of two primary proteins. The first major component is fibroin, consisting of a heavy chain (~390 kDa) and light chain, sericin, (~26 kDa) [7]. Silk fibroin can be constructed as particulate, two-dimensional (2D) or three-dimensional architecture (3D) [8]. Silk fibroin hydrogels (SF) are three-dimensional structure, which are provide congruous environment that is mimic extracellular

Correspondence author: Jeeeraphat Jantarat
Department of Operative Dentistry and Endodontics, Faculty of Dentistry, Mahidol University,
6 Yothi Road, Ratchathewi District Bangkok 10400 Thailand
Tel.: +662-200-7825-6 Fax : +662-200-7824 E-mail: jeeraphat.jan@mahidol.ac.th
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matrix in living tissue for cells and cytokines delivery [9]. Stem cells proliferation and differentiation has been tested with various types of silk fibroin scaffold, which reported different results [10-12]. The affecting factors such as scaffold fabrication methods, size and density of porous or even concentration of silk fibroin is influencing in cell proliferation and differentiation. Until now, there are only few studies of dental stem cells with silk fibroin scaffold have been published and none of the study use SF as a scaffold in their experiments [13, 14]. The SCAPs is also more clinically relevant to REPs than other stem cells. The objective of this study was to compare the efficiency of various concentrations of silk fibroin hydrogel scaffold for SCAPs proliferation.

Material and Methods

Cell culture

The SCAPs was transferred from the University of Texas Health Science Center at San Antonio [14]. Passages 5–6 of SCAPs were cultured in alpha-minimum essential medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Gemeni, West Sacramento, CA), 1 × L-glutamine (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich), and 100 U/mL streptomycin (Sigma-Aldrich), incubated at 37 °C and 5% CO2, and allowed to reach 80% confluence while changing media every 2 days.

Scaffold preparation

Silk fibroin was provided by Department of Biomedical Engineering, Mahidol University and was divided into 3 groups, according to concentration; 1.5% w/v, 2.0% w/v, and 2.5% w/v. The silk fibroin solution was activated using an ultrasonic device (P5 Newtrons®, Acteon, Mount Laurel, NJ) with modified ultrasonic tip for 1 min. Then activated silk fibroin solution was centrifuged at 1000 rpm for 10 sec.

Testing groups

The SCAPs at a concentration of 50,000 cells/sample was completely encapsulated in 1.5% w/v, 2.0% w/v, and 2.5% w/v silk fibroin hydrogel (SF) and transferred to gel mold. All samples were incubated at room temperature for 6–10 minutes in culture lamina flow hood for gel formation. The testing groups for SCAPs proliferation were placed in cell culture medium and the proliferation control group contains only silk fibroin hydrogel. The SCAPs encapsulated in silk fibroin hydrogel were incubated for 1, 7, 14 and 21days.

The SCAPs proliferation on silk fibroin hydrogel

The SCAPs proliferation on SF was assessed 1, 7, 14, and 21 days after culture using MTT assay (n=5). A colorimetric 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide metabolic assay (MTT, Sigma-Aldrich) was prepared at concentration of 1 mg/ml and added to 24-well plates and incubated for 4 hours at 37 °C. The MTT solution was then decanted, and 2 ml of isopropanol was added in each well. The plate was shaken for 60 minutes at room temperature to solubilize the formazan crystals. Two hundred microliters of isopropanol were transferred to 96 well–plate. A spectrophotometer was used to measure the absorbance values of wavelengths of 570 nm (RI Technologies, Bangkok, Thailand). Data were collected and compared by mean value of optical density.

Morphological analysis of SCAPs on SM by live/dead cell assay

Live/dead assay kit (Sigma-Aldrich) contained calcein-AM (green color) and propidium iodide (PI, red color) solutions, which stain viable and dead cells, respectively. The SCAPs on SF was cultured into culture media in 24-well plates, which was changed every 2 days. Live/dead assay was performed on Days 1, 7, 14, and 21 (n = 4). One milliliter of live/dead assay solution was added to each cultured well and it was incubated

at 37 °C for 15 minutes. Viable and dead cells morphologies were visualized using confocal microscope.

Statistical analysis

The difference of SCAPs proliferation in each day of experiment was analyzed using One-way ANOVA and Kruskal wallis test. Comparison between 1.5%, 2.0%, and 2.5% SF were performed by Tukey HSD and Dunn test. Significance was considered at 0.05 levels. Statistical analysis were conducted using the SPSS Statistics Version 18 (SPSS, Inc, USA).

Results

In vitro Proliferation of SCAPs on silk fibroin hydrogel

The proliferation of SCAPs cultured on various concentration of SF for up to 1, 7, 14, 21 days was carried out by MTT assay. The different of SCAPs proliferation in 3 various concentration of SF were compared by optical density as shown in Figure 1. The SCAPs growth on all SF concentration showed a slightly decreasing. On Day 7, SCAPs continues to proliferate on SF of concentration 1.5% and 2.5% at Day 14 and 21. However, SCAPs proliferation rate on SF at concentration of 2.0% exhibited increase on day 14 but decrease on day 21. The concentration of 1.5% SF show the highest SCAPs proliferation rate which significantly different when compared with 2.0% and 2.5% SF in all time points ($P<0.05$). The proliferation rate of SCAPs on 2.0% SF was higher than SCAPs on 2.5% SF. The significantly different of SCAPs proliferation between 2.0% and 2.5% SF was found of day 14 ($P<0.05$). The results suggested that 1.5% SF was the most suitable concentration for SCAPs proliferation

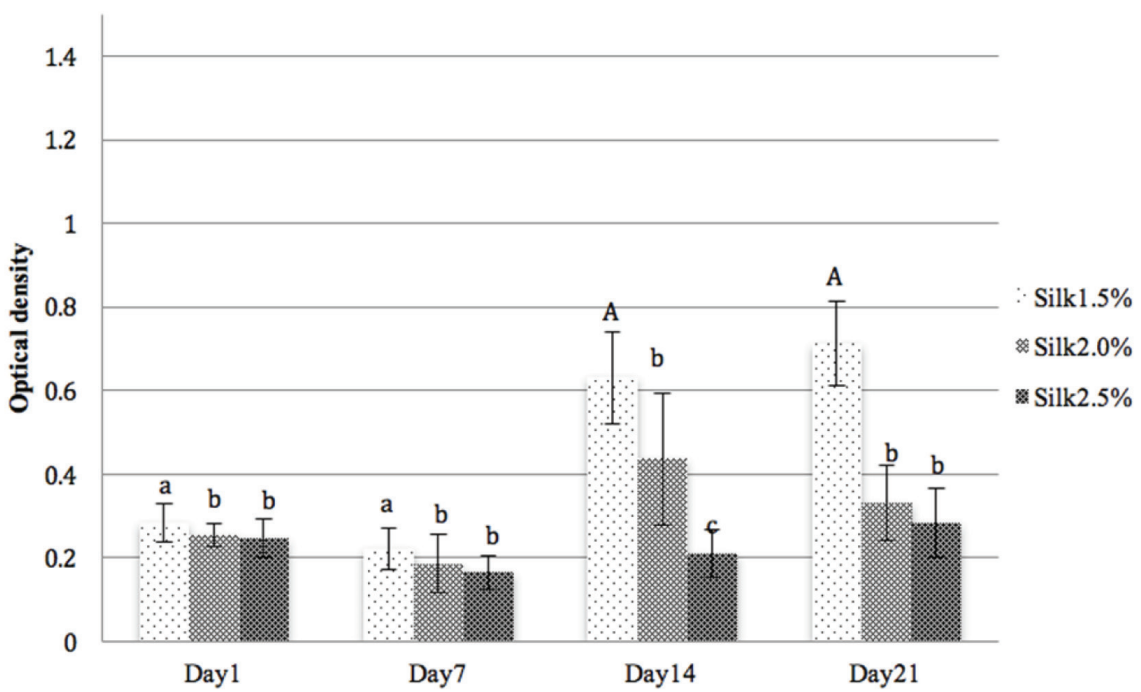

Figure 1 Proliferation of SCAPs cultured on SF for up to 1, 7, 14, and 21 days was reassured by MTT assay. The optical density value of SCAPs proliferation in various concentration of SF. Different letters represent statistically significant difference between each group of SF ($P<0.05$).

SCAPs morphology on silk fibroin hydrogel

The morphology of SCAPs on SF after culture for 1, 7, 14, 21 days was shown in Figure 2. The characteristic of SCAPs in all SF concentrations was round in shape on Day 1. On Day 7, SCAPs in 1.5% SF group exhibited starting the extension of cytoplasmic process, while the other groups did not. Cytoplasmic process of SCAPs on Day 14 and Day 21 on 1.5% SF was much more longer and interconnected with the neighboring cells comparing to the other concentrations (Figure 2).

The highest SCAPs proliferation was demonstrated in 1.5% SF and significantly different when compared with 2.0% and 2.5% SF on Day1, 7, 14 and 21 ($p<0.05$) (Figure 2).

Discussion

Silk fibroin liquid was formulated into hydrogel with ultrasound-sonication method. The unique technique for silk fibroin hydrogel gelation was developed using a routine equipment in endodontic treatment. Normally the completed blood clot occurs within 45-60 minutes. It does increase a chair-time in regenerative endodontic procedures. Thus, the gelation time for silk fibroin hydrogel scaffold should be minimized as low as possible. Our technique stimulated a completed setting of silk fibroin hydrogel scaffold within 10 minutes whereas previous studies took an hour to days [15-17]. Different concentration of silk fibroin in silk scaffold directly affects the macro/microstructure and mechanical properties of silk scaffold [11, 18, 19]. These can be caused of a diversity in cell proliferation and differentiation. Fabrication silk fibroin scaffold with various concentrations of silk fibroin has been showed different results on cell proliferation [11, 19]. Until now, the concentration of silk fibroin less than 1.5% w/v has not been tested and the concentration less than 1.5% w/v resulted in unstable and nonhomogeneous gel formation corresponding with the previous reported [20].

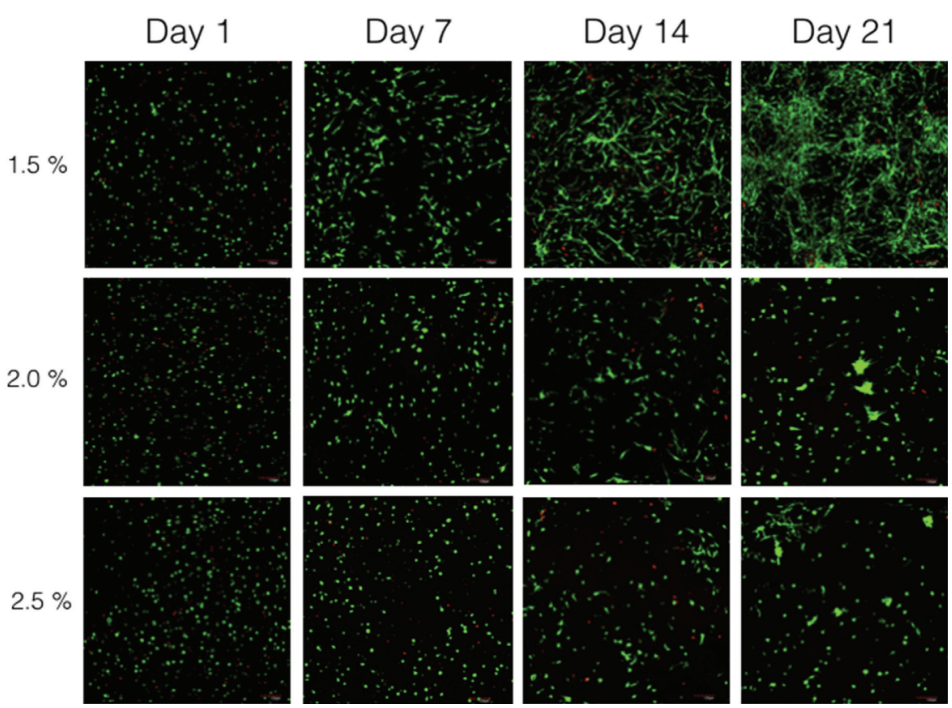

Figure 2 Representative immurotluoxscant images of stained SCAPs, Using live/dead assay kit. Cell morphology of SCAPs in 1.5%, 2.0% and 2.5% SF under confocal electron microscope on Day 1, 7, 14 and 21. Green spot represents live cell and red spot represents dead cell.

The MTT and Live/Dead assay were selected for evaluated SCAPs proliferation in our study. The results revealed, 1.5% SF had a highest SCAPs proliferation. The larger pore size, higher porosity and interconnection pore inside the lower concentration of silk fibroin scaffold which allow the exchange of oxygen and nutritional, are the possible explanations [11, 20]. In contrast with the higher silk fibroin concentration, cellular activity was diminished and led to cell dead when time passed. This situation results from mechanical restriction and decreasing rate of oxygen and nutritional diffusion [19]. The used of silk hydrogel scaffold has not been reported in dental treatment. This material offers many advantages. Fistly, it can be injected in to a root canal which make the REPs treatment more simple than creating a blood clot. Moreover, the setting time of silk fibroin scaffold activated in this experiment was only 10 minute which could reduce chair time of treatment. Finally, our research showed a promising result in term of non-toxic natural derived hydrogel scaffold supported SCAPs proliferation.

Conclusion

Silk fibroin hydrogel could be used as scaffold supported cell proliferation and 1.5% concentration illustrated a superior result in cell proliferation. However, further studies are required to investigated cell differentiation and the effect of microenvironment (in vivo) on cell and scaffold behavior.

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