Title: SILK FIBROIN HYDROGEL FOR SCAP PROLIFERATION AND DIFFERENTIATION

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Abstract	239	words
Total word count	2,286	words
Total number of tables	1	table
Total number of figures	3	figures
Total number of references	30	references

KEY WORDS: ODONTOBLAST / TISSUE ENGINEERING / STEM CELL(s) / PULP BIOLOGY / CELL DIFFERENTIATION / ENDODONTICS

SILK FIBROIN HYDROGEL FOR SCAP PROLIFERATION AND DIFFERENTIATION

ABSTRACT

Regenerative Endodontics is a treatment option of immature permanent teeth with apical periodontitis, which based on tissue engineering concept. Blood clot has been use as natural scaffold, however it is not always possible in every case. Since this problem has been recognized, researchers have begun examining for the new three-dimensional scaffold that can be substitute of blood clot. Silk fibroin hydrogels are three-dimensional structure, which provide congruous environment that is mimic extracellular matrix in living tissue for cells and cytokines delivery. The aim of this study was to compare the efficiency of various concentrations of silk fibroin hydrogel for SCAP proliferation and differentiation. Silk fibroin hydrogel was prepared at 1.5% w/v, 2.0% w/v and 2.5% w/v of silk fibroin concentration. The SCAP was successfully encapsulated in silk fibroin hydrogel and investigated SCAP proliferation using MTT and Live/dead assay. The odontogenic differentiation was evaluated using qRT-PCR for DMP-1, DSPP, MEPE and OCN gene expression with immunofluorescence straining for DSP. After day 1, 7, 14 and 21, 1.5% w/v silk fibroin hydrogel showed the highest SCAP proliferation. The expression of genes marker was found in all three-tested groups at all time points. The highest expression was exhibited in 2.0% w/v silk fibroin hydrogel group on day 21with a positive staining for DSP. It was concluded that 1.5% w/v silk fibroin hydrogel group had a superior property in SCAP proliferation, whereas, SCAP differentiation in 2.0% w/v exhibited much more odontoblast phenotype.

Introduction

The principle of regenerative endodontics 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, this step is not always possible 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 are three-dimensional structure, which are provide congruous environment that is mimic extracellular 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 (13, 14). There is no study on silk fibroin hydrogel as a scaffold in their experiments. The objective of this study is to compare the efficiency of various concentrations of silk fibroin hydrogel scaffold for Stem Cell of Apical Papilla (SCAP) proliferation.

Material and Methods

Cell culture

The SCAP has been identified as RP-89 (15), was transferred from University of Texas Health Science Center at San Antonio. Passage 5-6 of SCAP 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) and incubated at 37°C and 5% CO_2 and allowed to reach 80% confluence while changing media every 2 days.

Scaffold preparation

A mold to prepare the hydrogel silk scaffold was prepared by using a surgical blade to cut the top of 1 mL disposable syringe. The silk hydrogels were provided from Department of Biomedical Engineering, Mahidol University. According to silk fibroin concentration (SFC), the silk hydrogels will be divided into 3 groups; 1.5% w/v, 2.0% w/v and 2.5% w/v of silk fibroin concentration. The SCAP were prepared at a concentration of 10,000 cells/µl in an individual conical tube. The silk fibroin solutions were activated using ultrasonic device (P5 Newtrons®, Acteon, Mount Laurel, NJ) with modified ultrasonic tip for 1 min. After that, the activated silk fibroin solution was centrifuged at 1000 rpm for 10 sec. The SCAP and silk fibroin were mixed together and transferred to the gel mold. All samples were incubated at room temperature for 6-10 minutes in the culture lamina flow hood for gel formation.

The SCAP proliferation:

Live/dead cell assay

Live/dead assay (Sigma-Aldrich) contains Calcein-AM (green color) and Propidium Iodide (PI, red color) solutions, which stain viable and dead cells, respectively. Four replicate samples (silk fibroin hydrogel scaffold + SCAP) from each of the 3 SFC were evaluated. Samples were ejected from gel mold into culture media in 24 wells plate which was changed every 2 days. A live/dead assay was performed on days 0, 7, 14 and 21 (n=4 group/sample time for a total of 48 assays). The samples were added with 1 ml of assay solution and incubated at 37°C for 15 minutes. Viable and dead cells were visualized using a confocal microscope. Four images were captured per one sample. One image was randomly selected from each sample and analyzed using analytical suite imaging software (cellSens, Olympus, Japan). The percentage of live cells (P_{Live}) was measured by $P_{Live} = N_{Live}/(N_{Live}+N_{dead}) \times 100$, where N_{Live} is the number of life cell and N_{dead} is the number of dead cell in the same image. The live cell density (D) was measured as the percentage of specimen area that covered by live cells. The cell density was measured by $D = (N_{Live} / A_{Total})$, where N_{Live} is the area covered by live cells that stained green with calcein and A_{Total} is the total area of the field of view of the image.

MTT assay

At each time point of day 0, 7, 14 and 21, total 5 samples of each of the 3 experimental groups were analyzed. One hundred microliters of MTT (3-[4, 5- Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, Sigma-Aldrich) at a concentration of 1 mg/ml and added into 24 well-plate and incubated for 4 hours at 37° C. Then the MTT solution was decanted and 2 ml of isopropanol was added in each well. The plate was shaken for 60 minutes at the room temperature and transferred to 96-well plate spectrophotometer to read the absorbance. The data was calculated and compared by mean value of optical density.

The odontogenic differentiation

Silk fibroin hydrogel scaffold which was 1.5%, 2.0% and 2.5% SFC with SCAP, was placed in odontogenic induction medium consisting of 10 mmol/L β-glycerophosphate, 0.2 mmol/L ascorbate-2-phosphate, 100 mmol/L dexamethasone in alpha-minimum essential medium with 10% fetal bovine serum for 0, 7, 14 and 21 days. The odontogenic induction medium was replaced every 2 days.

qRT-PCR for dentin sialophosphoprotein (DSPP), dentin matrix protein (DMP-1), osteocalcin (OCN) and matrix extracellular phosphoglycoprotein (MEPE) gene expression

After 1, 7, 14, and 21 days of odontogenic induction, encapsulated SCAP in silk fibroin hydrogel was lysated and homogenized with beta-mercaptone (Sigma-Aldrich). Total RNA was extracted, using NucleoSpin® RNA Midi Kit (Macherey-Nagel, Duren) following the manufacturer's instruction. One microgram of mRNA of each sample was subjected to reverse transcription into cDNA using the iScript ™ Select cDNA Synthesiskit (BIO-RAD, Hercules, CA) according to the manufacturer's instruction. For qRT-PCRs, cDNA amplification was performed in the C1000™ thermal cycle of CFX96 real time system (BIO-RAD) using KAPA® Fast qPCR Kit (KAPABIOSYSTEMS, Wilmington, MA). Forty PCR cycles were performed with SYBR green I at 59.5 ^{o}C of the annealing temperatures. After PCR cycles ended, melting curve analysis was performed by heating to 95 ^{o}C for 10 seconds, followed by cooling to 65 ^{o}C for 5 seconds and gradual heating to 95 ^{o}C at 0.2 ^{o}C/second. The cDNA will be amplified with the following primers in Table 1. The expression of target genes was normalized with GAPDH,

and fold expression relative to control (undifferentiated SCAP) assessed by ΔΔCq method. All qRT-PCR were performed in triplicates.

Immunofluorescence staining for DSP

Encapsulated SCAP in silk fibroin hydrogel scaffold was confirmed for odontoblast differentiation under fluorescence microscope with immunostaining using polyclonal goat antihuman antibody to DSP (Santa Cruz Biotechnology, Dallas, Texas, 1:100 dilution) as the primary antibody and donkey anti-goat-IgG-FITC (Santa Cruz Biotechnology, 1:100 dilution) as a secondary antibody.

Data analysis

The difference of SCAP proliferation and differentiation in each day of experiment was analyzed using One-way ANOVA and Kruskal wallis test. Comparison between 1.5%, 2.0% and 2.5% SFC groups was performed by Tukey HSD and Dunn test. The level of significance was set at $P < 0.05$.

Results

SCAP proliferation

Live/Dead assay

The characteristic of SCAP in all silk fibroin hydrogel concentrations (Figure 1) were round in shape on day 1. On Day 7, SCAP in 1.5% SFC exhibited the extension of cytoplasmic process, while the other groups did not change from Day1. Cytoplasmic process of SCAP on Day 14 and Day 21 in 1.5% SFC was much more longer and interconnected with the neighboring cells comparing to the other concentrations. The percentage of SCAP viability (Figure 2A) in 1.5%, 2.0% and 2.5% was not different on Day1. After Day1, the percentage of SCAP viability was significantly decreased in 2.5% SFC ($p<0.05$) comparing with 1.5% and 2.0% SFC. The SCAP density (Figure 2B) in 1.5% SFC was significantly higher than the other groups ($p<0.05$) and increased at all time points of the experiment.

MTT assay

The increasing of SCAP proliferation was found on Day 14 and 21 except 2.0% SFC which was found a decreasing in SCAP proliferation on Day 21 (Figure 2C). The highest SCAP proliferation was demonstrated in 1.5% SFC and significantly different when compared with 2.0% and 2.5% SFC on Day1, 7, 14 and 21 ($p<0.05$)

SCAP differentiation

Gene expression during odontogenic differentiation of SCAP in 1.5%, 2.0% and 2.5% silk fibroin hydrogel scaffold.

The expression of DMP-1, DSPP and MEPE were slightly detected on Day 1, 7 and 14 in all tested silk fibroin concentration. Until Day 21, the highest expression of DMP-1, DSPP and MEPE were found in 2.0% SFC. The expression of OCN in 1.5% and 2.0% SFH were highest on day 14 follow by the decreasing Of OCN on Day 21. On the other hand, OCN expression in 2.0% SFC was increased from Day1 to Day 21 (Figure 3A, B, C and D).

The overall pattern of all genes both 1.5% and 2.5% were similar, a dramatically increasing of OCN on Day14, followed by a suddenly decreasing of OCN. A slightly expression was found on DMP-1, DSPP and MEPE. Different pattern of genes expression was demonstrated in 2.0% SFC. A continuously increasing of OCN from Day 1- Day 21 with a dramatically increased of MEPE, DSPP and DMP-1 on Day 21 (Figure 3E).

DSP protein expression by immunofluorescence

The blue color (DAPI) stained nucleus, whereas the green color stained DSP in cytoplasm of SCAPs. Positive staining for DSP was detected only on Day 21 in 2.0% SFC, while the other was not detected (Figure 4).

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 endodontics. 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 (16-18). Different concentration of silk fibroin in silk scaffold directly affects the macro/microstructure and mechanical properties of silk scaffold (11, 19, 20). 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, 20). Until now, the concentration of silk fibroin less than 1.5% has not been tested and the concentration less than 1.5% resulted in unstable and nonhomogeneous gel formation corresponding with the previous reported (21). The MTT and Live/Dead assay were selected for evaluated SCAP proliferation in our study. The results revealed, 1.5% SFC had a superior in cell 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 explanation (11, 21). 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 (20).

The ideal scaffold should support cell differentiation as well. Replacement of damage tooth structure including dentin and root structures, as well as cell of pulp-dentin complex is the purpose of regenerative endodontics. Thus, odontoblast is needed. Differentiation of odontoblast was investigated via the gene expression of DMP-1, DSPP, MEPE and OCN. A significant non-collagen protein, DSPP, which play a major role during dentin mineralization and highly express in odontoblast. It cleaves immediately into DSP and DPP and synthesizes during secretory phase of terminally differentiated odontoblast (22). The DMP-1 is an acidic phospholyrated extracellular matrix protein. It is expressed during the development of differentiating odontoblast and secreted into the extracellular matrix during the formation and maturation dentine (23, 24). Another gene involving in the regulation of cellular metabolism during mineralization processes is MEPE and it has been identified in dental tissue (25). The late stage of osteo/odotogenic differentiation marker is OCN that plays regulatory role in the mineral phase of bone and dentin (26). The result in our research found the expression of all genes was increasing during the day passed with the highest expression on Day 21 in 2% SFC, which was similar to previous study on gene expression during odontoblast differentiation (27, 28). Moreover, we also found the highly expression of DMP-1. The expression of DSPP and DMP-1 in our results was consistent with the hypothesis that both genes (DMP-1 and DSPP) play a role

in the mineralization of dentin and it was found in the early stage of odontogenesis (29). Surprisingly, OCN expression in 1.5% and 2.5% SFC was highest on Day 14 and dramatically decreasing was observed on Day 21 comparing with a few expressions of DSPP, DMP-1 and MEPE. These may imply that osteoblastic differentiation occurred on Day 14 because OCN was outstanding expression while the others, which is expressed by functional odontoblast, was slightly expression (23). According to our result qPCR, SCAP differentiation in 2.0% SFC was exhibited genes expression much more alike odontoblast, comparing with the other groups. A specific maker, DSP which is a principle protein extracellular matrix of tooth, was performed via immunofluorescence to emphasize the characteristic of mineralize tissue. A positive staining was found similar with the previous study (30). According to the result from qtPCR and immunofluorescence may imply that differentiated odontablast-liked cell was achieved on Day 21 in 2.0% SFC. This study concluded that a superior result in SCAP proliferation and odontogenic differentiation was found in 1.5% and 2.0% SFC, respectively. However, further experiment is needed to investigate the effect of microenvironment on cell and scaffold behavior.

Acknowledgement

This research has been supported by the Endodontics division, Faculty of Dentistry, Mahidol University and International Federation of Endodontic Associations (I.F.E.A) Jean-Marie Laurichesse Research Award.

The authors deny any conflicts of interest.

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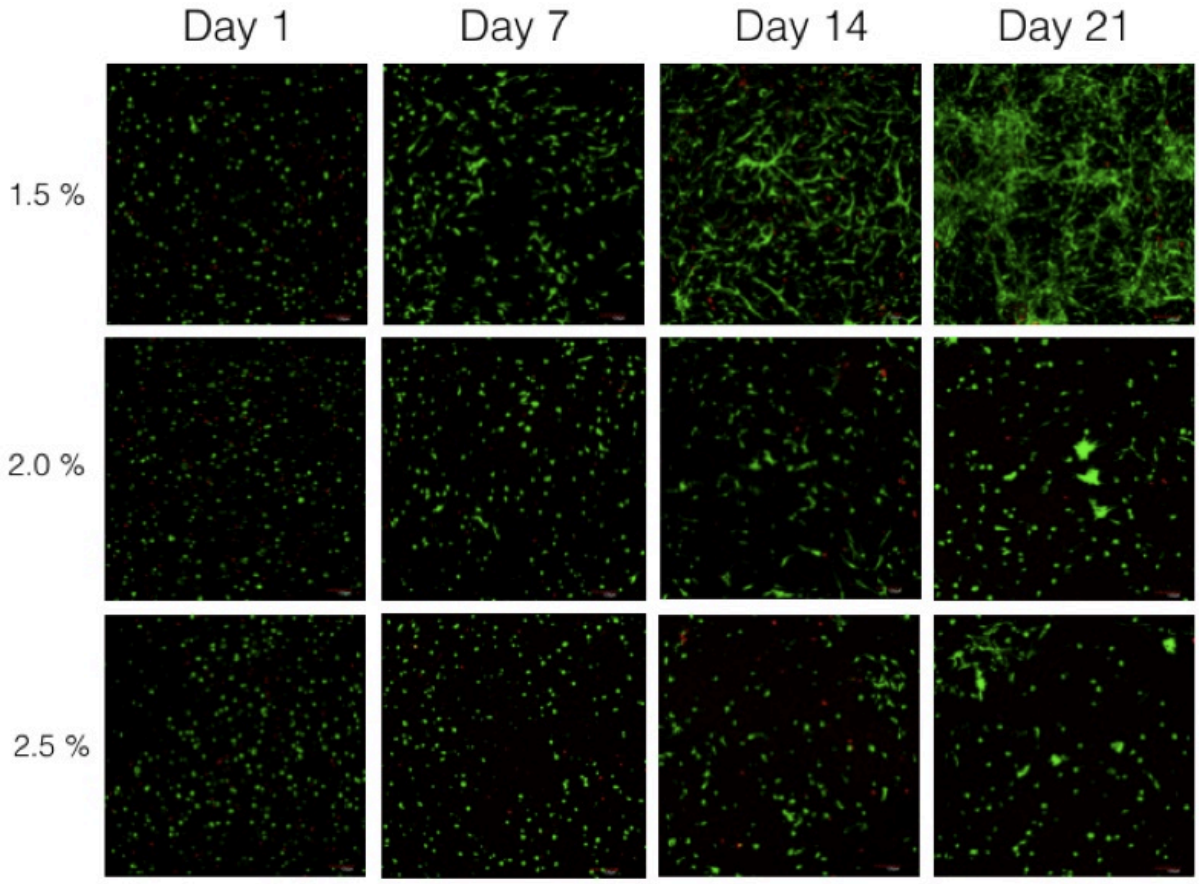

Figure 1. Cell morphology of SCAP in 1.5%, 2.0% and 2.5% SFC under confocal electron microscope on Day 1, 7, 14 and 21. Green spot represents live cell and red spot represents dead cell.

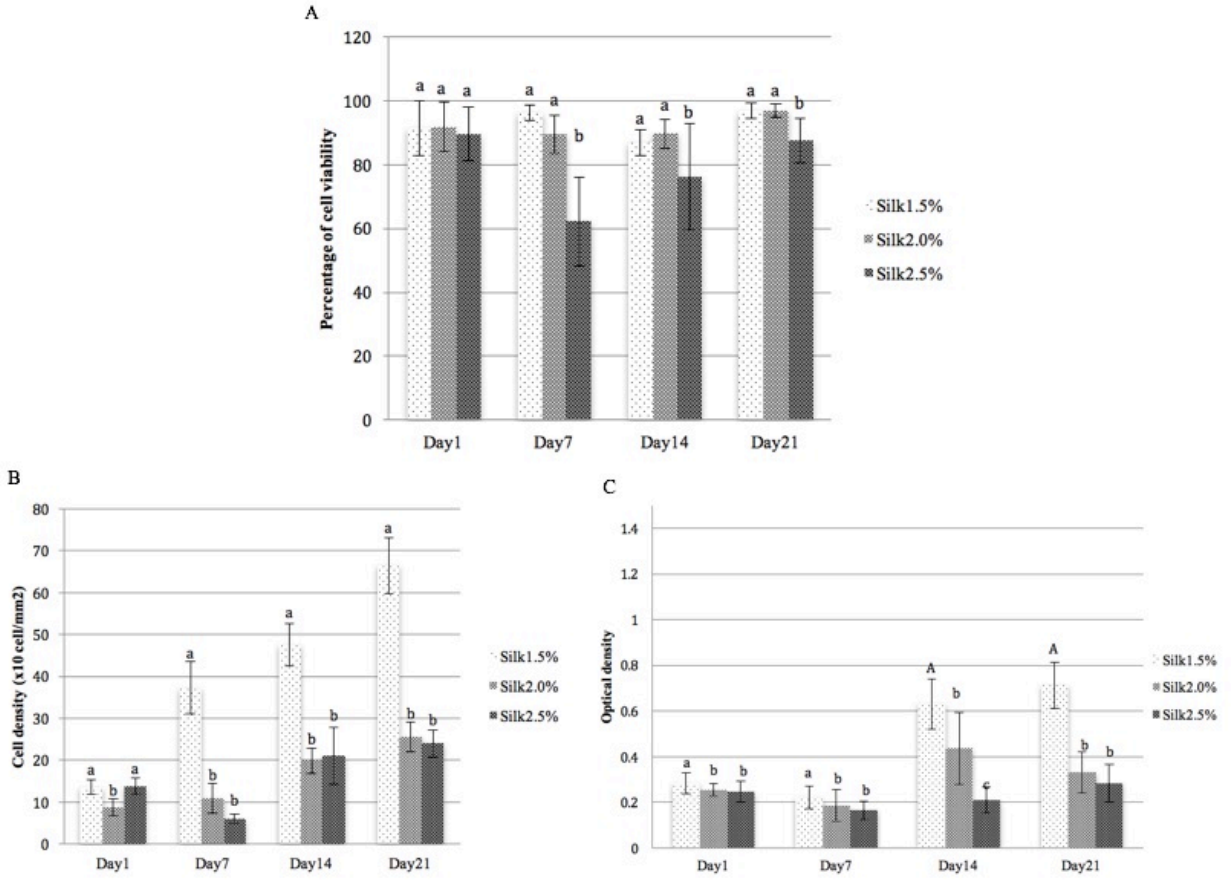

Figure 2. (A) The percentage of SCAP viability in various concentration of SFC. Different letters represent statistically significant difference between each group of SFC ($P<0.05$). (B) SCAP density in various concentration of SFC. Different letters represent statistically significant difference between each group of SFC ($P<0.05$). (C) The optical density value of SCAP proliferation in various concentration of SFC. Different letters represent statistically significant difference between each group of SFC ($P<0.05$).

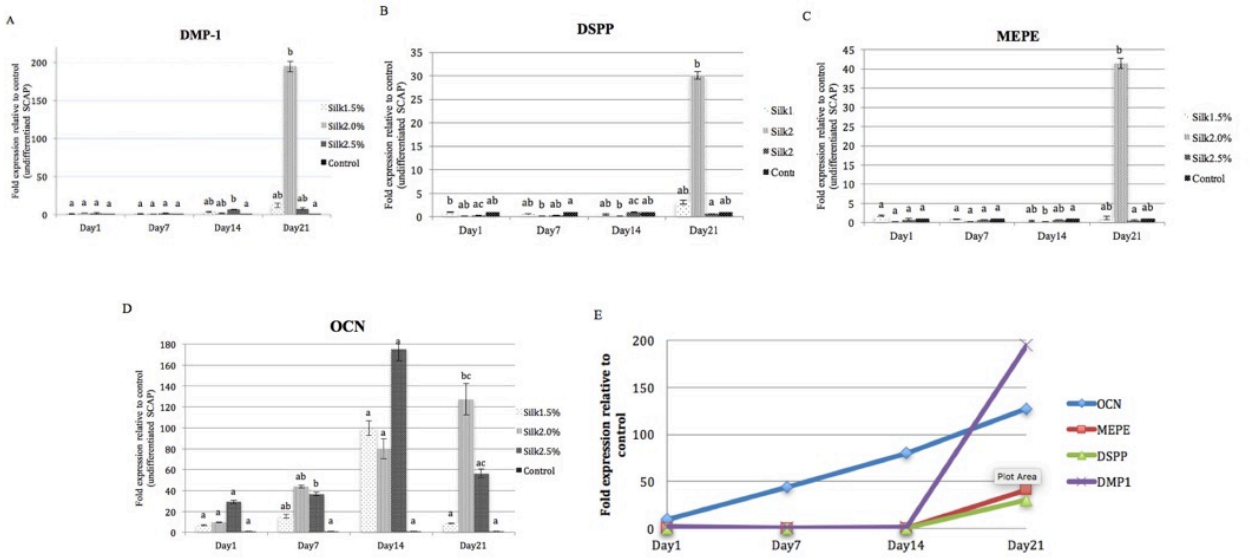

Figure 3. The means of normalized DMP-1, DSPP, MEPE and OCN gene expression (A, B, C and D). Different letters represent statistically significant difference between each group of SFC ($P<0.05$). The overall expression of all genes in 2.0% from Day 1 to Day 21(E)

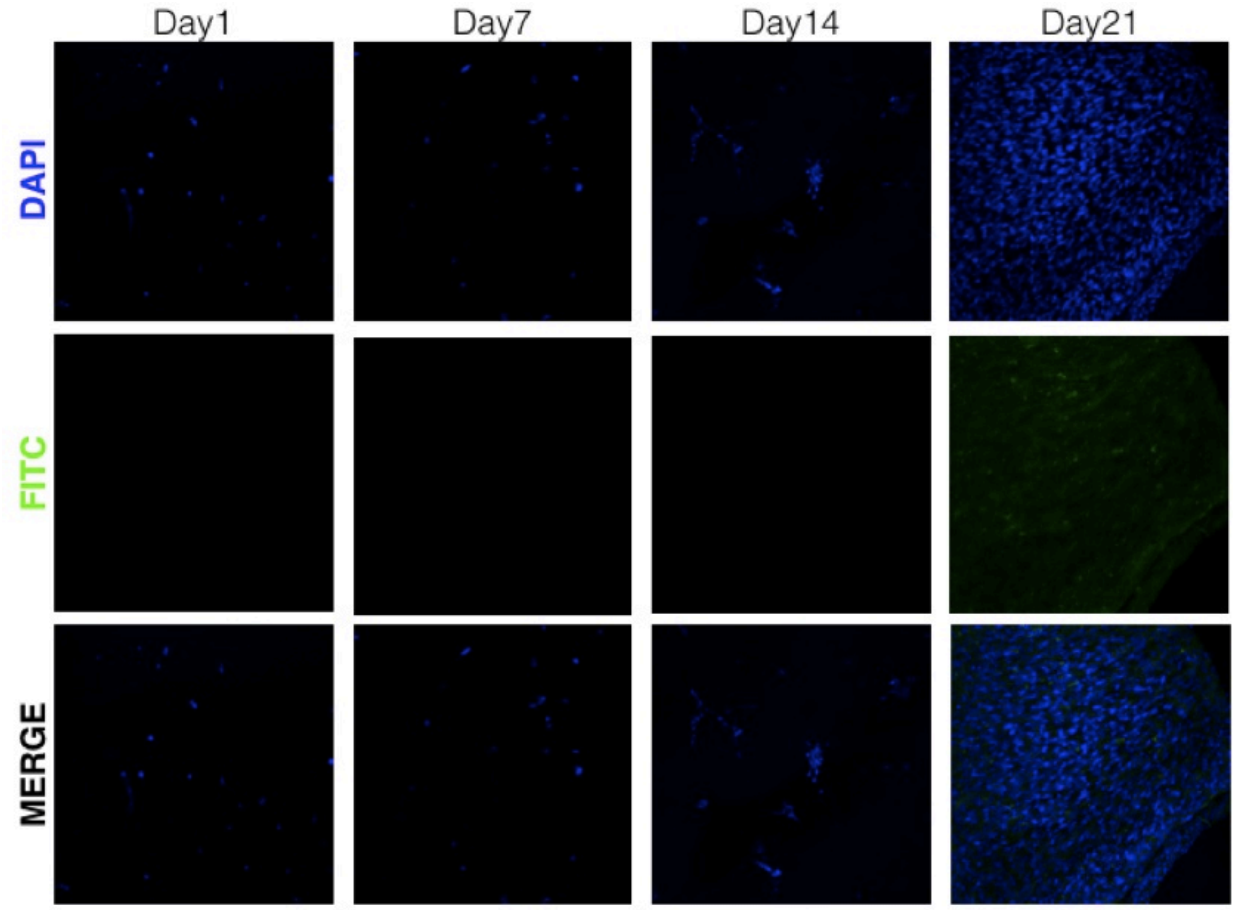

Figure 4. Immunofluorescence straining for DSP in 2.0% from Day 1 to Day 21. The positive staining of DSP was detected on Day 21.