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Brief communication (original)

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Osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells encapsulated in Thai silk fibroin/collagen hydrogel: a pilot study in vitro

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Abstract

Background: Silk fibroin (SF) can be processed into a hydrogel. SF/collagen hydrogel may be a suitable biomaterial for bone tissue engineering.

Objectives: To investigate in vitro biocompatibility and osteogenic potential of encapsulated rat bone marrow-derived mesenchymal stem cells (rat MSCs) in an injectable Thai SF/collagen hydrogel induced by oleic acid–poloxamer 188 surfactant mixture in an in vitro pilot study.

Methods: Rat MSCs were encapsulated in 3 groups of hydrogel scaffolds (SF, SF with 0.05% collagen [SF/0.05C], and SF with 0.1% collagen [SF/0.1C]) and cultured in a growth medium and an osteogenic induction medium. DNA, alkaline phosphatase (ALP) activity, and calcium were assayed at periodically for up to 5 weeks. After 6 weeks of culture the cells were analyzed by scanning electron microscopy and energy dispersive spectroscopy.

Results: Although SF hydrogel with collagen seems to have less efficiency to encapsulate rat MSCs, their plateau phase growth in all hydrogels was comparable. Inability to maintain cell viability as cell populations declined over 1–5 days was observed. Cell numbers then plateaued and were maintained until day 14 of culture. ALP activity and calcium content of rat MSCs in SF/collagen hydrogels were highest at day 21. An enhancing effect of collagen combined with the hydrogel was observed for proliferation and matrix formation; however, benefits of the combination on osteogenic differentiation and biomineralization are as yet unclear.

Conclusion: Rat MSCs in SF and SF/collagen hydrogels showed osteogenic differentiation. Accordingly, these hydrogels may serve as promising scaffolds for bone tissue engineering.

Keywords: collagen; fibroins; hydrogels; mesenchymal stem cells; silk

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Hydrogels are cross-linked hydrophilic polymer networks that can contain a high amount of water. Their hydrated network architecture provides a place for cells to adhere, proliferate, and differentiate. They are generally nontoxic and biocompatible. In tissue engineering, hydrogels can be used to encapsulate living cells as a cell delivery system and as a scaffold for tissue regeneration. Moreover, hydrogels can be delivered to a target site in a minimally invasive manner. Nevertheless, the gelling process must be benign for cell survival and to avoid damage to loaded cells [1].

Silk fibroin (SF), the structural protein in natural silkworm fibers, is a naturally derived polymer. Attributes of silk include good biocompatibility and slow biodegradability. In tissue engineering, silk has been widely studied in the design of various forms of scaffolds for regeneration of various tissues [2, 3]. SF can be processed into a hydrogel using time controlled chemical and physical methods, and makes it appropriate for the development of injectable in situ-forming hydrogels for bone tissue engineering [4–6]. Gelation of SF can be induced by surfactant and vortexing [7–9]. To apply in situ-forming SF hydrogel as a cell carrier in bone defect treatment, a suitable processing time is essential. SF gelation can be induced in 45–60 min by adding surfactants composed of a mixture of oleic acid and poloxamer-188 (poloxamer-188:oleic acid at 0.07:0.93 by weight), which is expected to be appropriate to prepare hydrogels to encapsulate cells, delivery to target sites, and set the gels in situ [10].

Collagen is a major protein in the extracellular matrix and plays an important role in supporting and strengthening the structural integrity of connective tissue. Due to its biocompatibility, relative lack of immunogenicity, nontoxicity, and promotion of cell adhesion, it is a promising biomaterial for tissue engineering.

Scaffolds made of combined SF and collagen have been developed to attain benefits of bioactivity from collagen and mechanical properties from the porous structure of SF. Various designs of SF/collagen scaffold have been explored for potential tissue engineering of various tissues [11–16]. Moreover, enhanced biomineralization has been demonstrated in coexistence with collagen and SF-derived polypeptide [17]. Therefore, hydrogels developed from a combination of collagen and SF could be expected to be beneficial in bone tissue engineering.

The aim of the present study was to evaluate the biocompatibility and osteogenic potential of rat mesenchymal stem cells (MSCs) encapsulated in injectable surfactant-induced Thai SF hydrogel supplemented with various concentrations of collagen compared with Thai SF hydrogel without added of collagen in cell culture in vitro.

Materials and methods

Cell isolation

Protocols using animals were approved by the Ethics Committee for Animal Care and Use of Chulalongkorn University (certificate of approval No. 06/2560) following The Animals for Scientific Purposes Act, BE 2558 (AD 2015), which regulates the use of both vertebrates and invertebrates, and conducted under license for Animals for Scientific Purposes from the Institute for Animals for Scientific Purpose Development and National Research Council of Thailand. MSCs were isolated from femurs of 3-week-old female Wistar rats obtained from a specialist supplier (. The femurs were dissected and cleaned with phosphate-buffered saline (PBS) before being cut at the proximal and distal ends. The bone marrow was extracted using a 24-G needle fitted to a syringe containing 1 mL of α-modified Eagle's medium (α-MEM) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The procedure was repeated until all of the bone marrow was harvested, and then the tissue was triturated by repeated aspiration through a 24-G needle. The resulting cell suspension was placed into a 75-cm^2 tissue culture flask containing 10 mL of medium and cultured in an incubator at 37°C, under an atmosphere of 5% CO_2 in air. The medium was changed on the fourth day and every 3 days thereafter [18].

When 80%–90% confluence was reached, the cells were released from the dish by treatment with trypsin–ethylene diamine tetraacetic acid (EDTA) and subcultured into a new set of culture dishes at 5×10^4 cells/cm^2 on the first passage. The cells from the first passage were again subcultured before they became confluent, and the cells from the second passage were used for experiments.

Scaffold preparation and cell encapsulation

Pupae-free Thai silk cocoons from *Bombyx mori* (Nangnoi-Srisaket 1) from the Queen Sirikit Sericulture Center (Nakhon Ratchasima province, Thailand) were used to prepare sterile aqueous SF solution by a method modified from Ratanavaraporn et al. [19]. About 40 g of the pupae-free Thai silk cocoons was added to 1 L of boiling 0.02 M sodium carbonate (Na_2CO_3) solution for 20 min. The fibers were then rinsed thoroughly with deionized water (DI) to remove sticky gum protein glue, sericin, from the fibroin filaments. The degummed silk fibers were air dried at room temperature. Then dried fibers were sterilized by autoclaving at 121°C for 15 min. The sterilized fibers were dissolved in 9.3 M LiBr at 60°C for 4 h with 4 g:16 mL silk:LiBr. The solution was dialyzed against

sterile DI water for 48 h to remove the LiBr. The SF solution obtained was then centrifuged at 9,000 rpm at 4°C for 20 min to remove insoluble parts. The final concentration of regenerated aqueous SF solution was about 5.5%–6.0% wt. All equipment was sterilized before use, and the whole process was performed under sterile conditions. The sterility of aqueous SF solution was proved by microbiological attribution tests including a total plate count and total yeast and molds [10].

Hydrogels were prepared from sterile SF solution (4% wt) with or without sterile collagen solution (Nitta Gelatin) and allocated to 3 groups: group 1, hydrogels made of 4% SF solution only (SF hydrogel), group 2, hydrogels made of 4% SF solution + 0.05% collagen solution (SF/0.05C hydrogel), and group 3, hydrogels made of 4% SF solution + 0.1% collagen solution (SF/0.1C hydrogel). The gelation was induced by the addition of surfactants (poloxamer-188:oleic acid at 0.07:0.93 by weight). Then each sample was mixed on a vortex mixer for 90 s before incubation at 37°C for 30–45 min.

Cell viability and proliferation

The cells treated with trypsin and resuspended in α-MEM to obtain a cell density of 5×10^6 cell/mL. Then 100 µl of the cell suspension was added and mixed with the vortexed silk solution giving a final concentration of 5×10^5 cells/mL. An aliquot of 0.5 mL of the mixtures was quickly pipetted into a stainless-steel ring placed in a 24-well cell culture plate and incubated at 37°C, under an atmosphere of 5% CO_2 in air. A hydrogel formed within 45–60 min. Then the rings were removed to obtain cylindrical-shaped gels with 12 mm diameter and 5 mm thick.

All samples were then cultured in 2 mL of growth medium containing α-MEM supplemented with 15% FBS and 1% penicillin–streptomycin at 37°C under an atmosphere of 5% CO_2 in air. The medium was refreshed every 2 days. The number of cells was analyzed by DNA assay at 6 h, 1 day, 3 days, 5 days, 7 days, 10 days, and 14 days. The hydrogel was minced and added to 1 mL sodium dodecyl sulfate (SDS) lysis buffer and incubated at 37°C under an atmosphere of 5% CO_2 in air for 1 h to disrupt cell membranes. The obtained cell lysates were then centrifuged to separate supernatants. About 100 µL of supernatant was pipetted into a 96-well black plate, and then Hoechst 33258 reagent (20 µL Hoechst solution + 19 mL DI + 1 mL saline-sodium citrate) was added. The fluorescent intensity of the solution at 355 nm (excitation) and 460 nm (emission) was measured immediately using a microplate reading fluorometer and compared with a standard curve indicating cell numbers. The efficiency of rat MSC encapsulation was calculated at various times from 6 h.

Osteogenic differentiation

Rat MSCs (1×10^6 cells/mL) were cultured in osteogenic induction medium consisting of α-MEM supplemented with 10% FBS, 1% penicillin–streptomycin, 50 µg/mL L-ascorbic acid, 10^{-8} M dexamethasone, and 10^{-2} M β-glycerol phosphate and then incubated at 37°C under an atmosphere of 5% CO_2 in air. The medium was changed twice a week. The osteogenic differentiation of MSC was determined by DNA assay, alkaline phosphatase (ALP) activity assay, and calcium assay at 3 days, 7 days, 14 days, 21 days, 28 days, and 35 days.

ALP activity assay

Minced hydrogel was added to 1 mL of SDS and incubated at 37°C under an atmosphere of 5% CO_2 in air for 1 h to disrupt cell membranes and then 20 µL samples pipetted into wells of a 96-well plate. *p*-Nitrophenyl phosphate (100 µL) was added to the cell lysates in each well and then together incubated at 37°C under an atmosphere of 5% CO_2 for 15 min. The reaction was stopped with 80 µL of 0.02 N NaOH, and *p*-nitrophenol, which is a product of hydrolysis of *p*-nitrophenol phosphate catalyzed by ALP, was measured using a microplate reading spectrophotometer at 405 nm [20].

Calcium assay

Minced hydrogel was added to 1 mL SDS and incubated at 37°C under an atmosphere of 5% CO_2 in air for 1 h to disrupt cell membranes and then 100 µL samples pipetted into wells of a 48-well plate. 1 M HCl (100 µL) was added to the cell lysates and the mixture incubated at 37°C under an atmosphere of 5% CO_2 in air for 4 h to extract calcium. Aliquots (10 µl) of the cell lysate extracts were pipetted into wells of a 48-well plate. Ethanolamine buffer (1 mL of 0.88 M) and *o*-cresolphthalein complex substrate (OCPC) (100 µl of 0.63 M) were mixed together with the sample in each well of the plates. The reaction between OCPC and calcium yielded calcium-*o*-cresolphthalein complex measured using a microplate reading spectrophotometer at 570 nm [21].

Scanning electron microscopy and energy dispersive spectroscopy

After rat MSCs had been cultured in hydrogels for 6 weeks, hydrogels were cut into slices and dehydrated in a graded series of ethanol concentrations before drying with a critical point dryer. The dried specimens were sputtered with gold

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using a fine coater. The specimens were then examined using digital scanning electron microscopy (SEM; JSM-6610LV; Jeol). To determine the elemental composition of the specimens, the energy dispersive spectroscopy (EDS; 10–30 kV X-Max^N, Oxford Instruments) spectrum was plotted and analyzed.

Statistical evaluation

All experiments were performed in triplicate. The results are reported as mean ± standard deviation (SD). Hydrogels were compared using a one-way analysis of variance (ANOVA) with a post hoc least significant difference (LSD) test. The level of significance was set at $P < 0.05$.

Results

Cell viability and proliferation

The results of the DNA assay at 6 h revealed that the encapsulation efficiency of rat MSCs in SF hydrogels was 18.20 ± 3.21 ($\times 10^4$ cells), in SF/0.05C hydrogels was 14.03 ± 0.06 ($\times 10^4$ cells), and in SF/0.1C hydrogels was 10.74 ± 3.24 ($\times 10^4$ cells). Rat MSCs proliferated on all hydrogels in the first 24 h, and then cell numbers declined before reaching plateau phase at 5 days. Subsequently, the number of cells encapsulated was maintained during days 5–14 of culture. The MSC cultured on SF/0.1C hydrogel exhibited the lowest encapsulation efficiency and cell numbers during day 3–5, but when the cultured cells reach plateau phase during days 7–14, there were no significant differences in cell numbers among the various hydrogels (**Figure 1**).

Osteogenic differentiation

At 21 days, the cell proliferation in SF/0.1C hydrogels was significantly greater than in SF hydrogels. We found were no significant differences observed between the 3 groups of hydrogels at other times (**Figure 2**).

After 1 week of culture, ALP was found increased in MSCs cultured in all hydrogels. Then the ALP level in SF/0.1C hydrogel decreased, whereas in SF and SF/0.05C hydrogels, the activity continued increasing to the highest level at 3 weeks (**Figure 3**).

Calcium level in SF and SF/0.05C hydrogels was significantly higher in SF/0.1C hydrogel at 21 days, but no differences were seen in other time points (**Figure 4**).

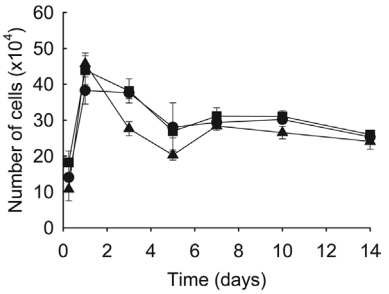

Figure 1. Growth kinetics of rat mesenchymal stem cells cultured in hydrogels. Squares indicate hydrogels made of 4% silk fibroin (SF) solution only (SF hydrogel), circles indicate hydrogels made of 4% SF solution + 0.05% collagen solution (SF/0.05C hydrogel), and triangles indicate hydrogels made of 4% SF solution + 0.1% collagen solution (SF/0.1C hydrogel). Error bars are standard deviations of triplicate experiments.

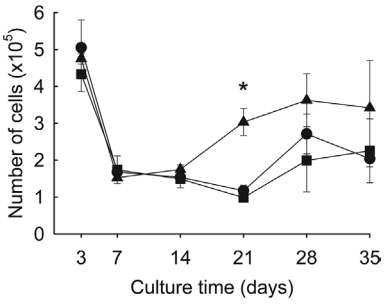

Figure 2. DNA assay of rat mesenchymal stem cells cultured in hydrogels in osteogenic induction medium. Squares indicate hydrogels made of 4% silk fibroin (SF) solution only (SF hydrogel), circles indicate hydrogels made of 4% SF solution + 0.05% collagen solution (SF/0.05C hydrogel), and triangles indicate hydrogels made of 4% SF solution + 0.1% collagen solution (SF/0.1C hydrogel). *$P < 0.05$ (one-way analysis of variance with a post hoc least significant difference test). Error bars are standard deviations of triplicate experiments.

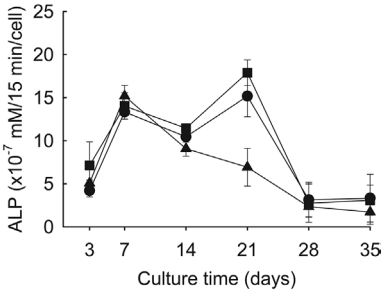

Figure 3. Alkaline phosphatase (ALP) activity of rat mesenchymal stem cells cultured on hydrogels in osteogenic induction medium. Units indicate mM *p*-nitrophenol produced per min per cell. Squares indicate hydrogels made of 4% silk fibroin (SF) solution only (SF hydrogel), circles indicate hydrogels made of 4% SF solution + 0.05% collagen solution (SF/0.05C hydrogel), and triangles indicate hydrogels made of 4% SF solution + 0.1% collagen solution (SF/0.1C hydrogel). Error bars are standard deviations of triplicate experiments.

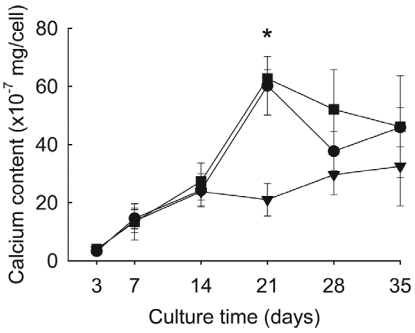

Figure 4. Calcium content of rat mesenchymal stem cells cultured on hydrogels in osteogenic induction medium. Squares indicate hydrogels made of 4% silk fibroin (SF) solution only (SF hydrogel), circles indicate hydrogels made of 4% SF solution + 0.05% collagen solution (SF/0.05C hydrogel), and triangles indicate hydrogels made of 4% SF solution + 0.1% collagen solution (SF/0.1C hydrogel).
*P < 0.05 (one-way analysis of variance with a post hoc least significant difference test). Error bars are standard deviations of triplicate experiments.

SEM and EDS

SEM analysis revealed random distribution of osteoblast-like cells within the pores of scaffold in all hydrogels. Nevertheless, the culture in SF/0.1C hydrogel showed most abundant fibril-like matrix, whereas the SF hydrogel exhibited least matrix amount (**Figure 5**). EDS analysis showed the presented amount of calcium level in the following order: SF/0.05C > SF > SF/0.1C. The Ca/P ratios of SF, SF/0.05C, and SF/0.1C were 4.28 ± 2.38, 2.91 ± 1.09, and 3.61 ± 1.75, respectively (**Table 1**).

Discussion

Development of an injectable hydrogel made of Thai SF and collagen theoretically provides benefits from bioactivity of

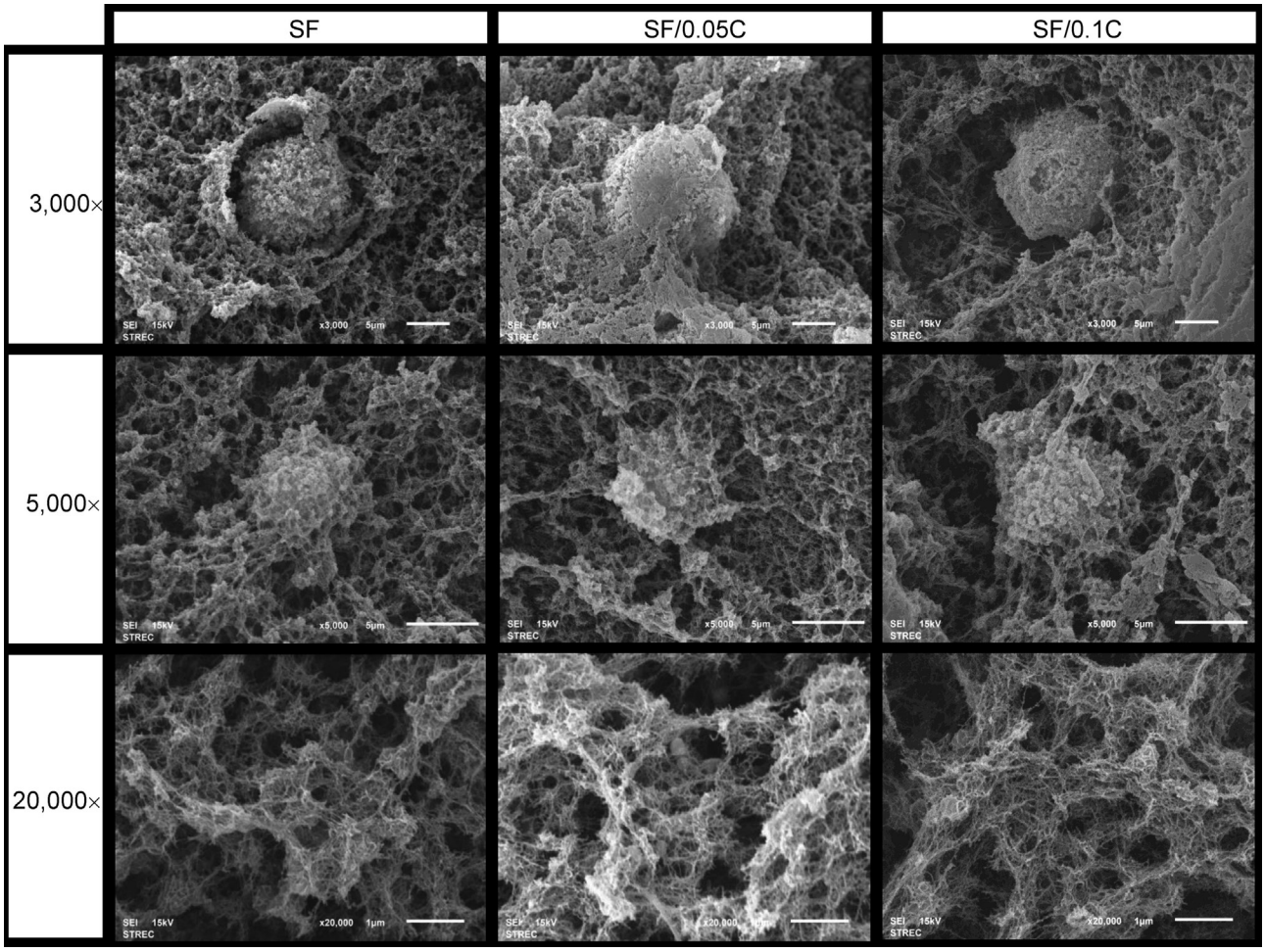

Figure 5. SEM images of rat MSCs cultured in hydrogels under different magnifications. SF (hydrogel made of 4% silk fibroin (SF) solution only, SF/0.05C (hydrogel made of 4% SF solution + 0.05% collagen solution), and SF/0.1C (hydrogel made of 4% SF solution + 0.1% collagen solution).

Table 1. Elemental analysis result by EDS

Hydrogels	C (% wt)	O (% wt)	Ca (% wt)	P (% wt)	Ca/P
SF	45.03 ± 5.56	37.61 ± 3.75	13.99 ± 1.68	4.03 ± 1.81	4.28 ± 2.38
SF/0.05C	39.36 ± 2.96	33.99 ± 10.56	19.27 ± 7.13	7.38 ± 3.37	2.91 ± 1.09
SF/0.1C	45.92 ± 10.00	38.96 ± 3.11	10.86 ± 6.04	4.27 ± 4.37	3.61 ± 1.75

collagen and mechanical properties with porous structure from SF, which are suitable for the use in bone tissue engineering. Inducing an appropriate gelation time for this hydrogel for proper cell loading and delivery without toxicity or damage to loaded cells is essential. In the present study, we controlled the gelation time of SF solution to be 45–60 min by adding a surfactant composed of a mixture of oleic acid and poloxamer-188 and vortexing [10]. Combining these two methods created shorter gelation time using less surfactant and a shorter vortexing time. This probably resulted in a more benign milieu for survival and differentiation of the loaded cells than using each gel induction method separately.

The viability and proliferation of encapsulated rat MSCs demonstrated in the present study verified the biocompatibility of SF and combined SF/collagen hydrogel scaffolds. Even though the SF hydrogel with collagen seems to have less encapsulation efficiency for rat MSCs, the growth at the plateau phase of all hydrogels was comparable. We observed that cell viability declined during days 1–5. Cell numbers then plateaued and were maintained until day 14 of culture. This indicated a balance between apoptotic cell death and proliferation similar as found in culture of human MSCs in a chitosan–hyaluronan-based hydrogel in vitro [22]. The cell density at plateau phase was approximately 20%–30% of loaded cells. The viability of rat MSCs in SF and SF/collagen hydrogels presented in the present study was lower than that reported for other hydrogels [23–25]. Factors contributing to loss of viability of rat MSCs in SF and SF/collagen hydrogels may include cell damage from surfactants, limited cell distribution in the hydrogel, inadequate mass transport, or partial degradation of the hydrogel.

The highest ALP activity and calcium content detected in rat MSCs cultured in SF and SF/0.05C hydrogels was at 3 weeks significantly demonstrated the potential of the gels to allow induction of osteogenic differentiation of encapsulated rat MSCs in induction medium even with the apparent initial cell loss and the low proliferation. The rat MSC growth kinetics in the induction medium revealed the same characteristics as those in the growth medium. The cell numbers decreased in the first week before maintaining their level in the second week. From the third week, there was greater proliferation of rat MSCs in SF/0.1C than in SF and SF/0.05C hydrogels. By contrast, SF/0.1C showed less ALP activity and calcium content, indicating less osteogenic differentiation. According to these findings, SF/0.1C appeared to express a greater promotional effect on proliferation than differentiation than the SF and SF/0.05C hydrogels. This finding does not support the proposal of enhanced biomineralization with combined SF and collagen in hydrogels. We postulate that in blended SF and collagen hydrogels, not only the biological activity of collagen results in varied osteogenic differentiation in each hydrogel, but also the physicochemical properties of the hydrogels, such as density, stiffness, hydrophobicity, gel stability, and water absorption, are altered due to the addition of collagen, and must also be taken into account. Further studies on pre- and postencapsulated rat MSCs in hydrogels are warranted.

Comparable distributions of osteoblast-like cells within the pores of the scaffold were observed by SEM of each hydrogel group. However, a greater amount of matrix was noted in SF hydrogels supplemented with collagen. This finding indicates the beneficial effect of collagen when inducing matrix formation.

Calcium content analyzed by EDS was highest in the SF/0.05C hydrogel, followed by that in the SF hydrogel, and was lowest in SF/0.1C. This observation appeared to be correlated with the results of ALP activity and calcium assay, which does not meet the expectation of facilitated mineralization in combined SF and collagen hydrogel. Compared with the Ca/P ratio of hydroxyapatite and calcium phosphate (1.67–2.0) [17], the Ca/P ratio found in this study was around 2.9–4.3, suggesting that other forms of calcium compounds presented in the hydrogels during the osteogenic stimulus.

Conclusion

The injectable Thai SF and SF/collagen hydrogel scaffolds induced by surfactant demonstrated biocompatibility in cell encapsulation and the potential for allowing osteogenic differentiation of rat MSCs. Collagen combined in the hydrogel yielded a positive effect on proliferation and matrix formation; however, benefits on osteogenic differentiation and biomineralization were not demonstrated as expected. We conclude that these hydrogels could be used as scaffolds to promote bone regeneration in situ. However, further study of their physicochemical properties, biodegradation, and more information regarding their ability to allow or promote osteogenic differentiation is suggested to obtain a better understanding

to optimize this system as a scaffold and cell carrier in bone tissue engineering.

Author contributions. JA, SKU, and JJ contributed substantially to the conception and design of this study. JA, SKU, and SKA substantially acquired the data and JA, SH, and JJ analyzed and interpreted it. JA, SH, and JJ drafted the manuscript and SKU and SKA critically revised it. All the authors approved the final version submitted for publication and take responsibility for statements made in the published article.

Conflicts of interest statement. The authors have each completed and submitted an International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflicts of interest.

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References

[1] Nicodemus GD, Bryant SJ. Cell encapsulation in biodegradable hydrogels for tissue engineering applications. Tissue Eng Part B Rev. 2008; 14:149–65.

[2] Thitiwuthikiat P, Ii M, Saito T, Asahi M, Kanokpanont S, Tabata Y. A vascular patch prepared from Thai silk fibroin and gelatin hydrogel incorporating simvastatin-micelles to recruit endothelial progenitor cells. Tissue Eng Part A. 2015; 21:1309–19.

[3] Vorrapakdee R, Kanokpanont S, Ratanavaraporn J, Waikakul S, Charoenlap C, Damrongsakkul S. Modification of human cancellous bone using Thai silk fibroin and gelatin for enhanced osteoconductive potential. J Mater Sci Mater Med. 2013; 24:735–44.

[4] Floren M, Migliaresi C, Motta A. Processing techniques and applications of silk hydrogels in bioengineering. J Funct Biomater. 2016; 7:pii:E26. doi: 10.3390/jfb7030026

[5] Wang HY, Zhang YQ. Processing silk hydrogel and its applications in biomedical materials. Biotechnol Prog. 2015; 31:630–40.

[6] Rockwood DN, Preda RC, Yücel T, Wang X, Lovett ML, Kaplan DL. Materials fabrication from *Bombyx mori* silk fibroin. Nat Protoc. 2011; 6:1612–31.

[7] Kang GD, Nahm JH, Park JS, Moon JY, Cho CS, Yeo JH. Effects of poloxamer on the gelation of silk fibroin. Macromol Rapid Commun. 2000; 21:788–91.

[8] Wu X, Hou J, Li M, Wang J, Kaplan DL, Lu S. Sodium dodecyl sulfate-induced rapid gelation of silk fibroin. Acta Biomater. 2012; 8:2185–92.

[9] Yücel T, Cebe P, Kaplan DL. Vortex-induced injectable silk fibroin hydrogels. Biophys J. 2009; 97:2044–50.

[10] Apinun J, Yamdech R, Damrongsakkul S, Jamkratoke J, Kanokpanont S. Viability of rat's bone marrow-derived mesenchymal stem cells in a surfactant-induced Thai silk fibroin hydrogel. In: Biomedical Engineering International Conference: BMEiCON 2017: Proceedings of the 10th Biomedical Engineering International Conference; 2017 31 Aug – 2 Sept; Hokkaido, Japan.

[11] Ghezzi CE, Marelli B, Donelli I, Alessandrino A, Freddi G, Nazhat SN. The role of physiological mechanical cues on mesenchymal stem cell differentiation in an airway tract-like dense collagen-silk fibroin construct. Biomaterials. 2014; 35:6236–47.

[12] Ghezzi CE, Marelli B, Donelli I, Alessandrino A, Freddi G, Nazhat SN. Multilayered dense collagen-silk fibroin hybrid: a platform for mesenchymal stem cell differentiation towards chondrogenic and osteogenic lineages. J Tissue Eng Regen Med. 2015; 11:2046–59.

[13] Long K, Liu Y, Li W, Wang L, Liu S, Wang Y, Ren L. Improving the mechanical properties of collagen-based membranes using silk fibroin for corneal tissue engineering. J Biomed Mater Res A. 2015; 103:1159–68.

[14] Shen Y, Redmond SL, Papadimitriou JM, Teh BM, Yan S, Wang Y, et al. The biocompatibility of silk fibroin and acellular collagen scaffolds for tissue engineering in the ear. Biomed Mater. 2014; 9:015015. doi: 10.1088/1748-6041/9/1/015015

[15] Wang G, Hu X, Lin W, Dong C, Wu H. Electrospun PLGA-silk fibroin-collagen nanofibrous scaffolds for nerve tissue engineering. In Vitro Cell Dev Biol Anim. 2011; 47:234–40.

[16] Wei G, Li C, Fu Q, Xu Y, Li H. Preparation of PCL/silk fibroin/collagen electrospun fiber for urethral reconstruction. Int Urol Nephrol. 2015; 47:95–9.

[17] Marelli B, Ghezzi CE, Alessandrino A, Barralet JE, Freddi G, Nazhat SN. Silk fibroin derived polypeptide-induced biomineralization of collagen. Biomaterials. 2012; 33:102–8.

[18] Vachiraroj N, Ratanavaraporn J, Damrongsakkul S, Pichyangkura R, Banaprasert T, Kanokpanont S. A comparison of Thai silk fibroin-based and chitosan-based materials on in vitro biocompatibility for bone substitutes. Int J Biol Macromol. 2009; 45:470–7.

[19] Ratanavaraporn J, Kanokpanont S, Damrongsakkul S. The development of injectable gelatin/silk fibroin microspheres for the dual delivery of curcumin and piperine. J Mater Sci Mater Med. 2014; 25:401–10.

[20] Ginis I, Weinreb M, Abramov N, Shinar D, Merchav S, Schwartz A, et al. Bone progenitors produced by direct osteogenic differentiation of the unprocessed bone marrow demonstrate high osteogenic potential in vitro and in vivo. Biores Open Access. 2012; 1:69–78.

[21] Takahashi Y, Yamamoto M, Tabata Y. Osteogenic differentiation of mesenchymal stem cells in biodegradable sponges composed of gelatin and β-tricalcium phosphate. Biomaterials. 2005; 26:3587–96.

[22] Lindborg BA, Brekke JH, Scott CM, Chai YW, Ulrich C, Sandquist L, et al. A chitosan-hyaluronan-based hydrogel-hydrocolloid supports *in vitro* culture and differentiation of human mesenchymal stem/stromal cells. Tissue Eng Part A. 2015; 21:1952–62.

[23] Moshaverinia A, Chen C, Akiyama K, Xu X, Chee WW, Schricker SR, Shi S. Encapsulated dental-derived mesenchymal stem cells in an injectable and biodegradable scaffold for applications in bone tissue engineering. J Biomed Mater Res A. 2013; 101:3285–94.

[24] Raucci MG, Alvarez-Perez MA, Demitri C, Sannino A, Ambrosio L. Proliferation and osteoblastic differentiation of hMSCs on cellulose-based hydrogels. J Appl Biomater Funct Mater. 2012; 10:302–7.

[25] Sánchez-Ferrero A, Mata Á, Mateos-Timoneda MA, Rodríguez-Cabello JC, Alonso M, Planell J, Engel E. Development of tailored and self-mineralizing citric acid-crosslinked hydrogels for in situ bone regeneration. Biomaterials. 2015; 68:42–53.