Original Article

Evaluation of Bone Regeneration Using Injectable Surfactant-Induced Thai Silk Fibroin/Collagen In Situ-Forming Hydrogel in Segmental Bone Defects in Rats

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Objective: To evaluate an injectable Thai silk fibroin/collagen in situ-forming hydrogel induced by surfactant as a carrier to encapsulate cell and growth factor for bone regeneration.

Materials and Methods: Silk fibroin/collagen hydrogels induced by a surfactant mixture of oleic acid and poloxamer-188, both with and without rat mesenchymal stem cells (MSCs) and/or human platelet-rich plasma (PRP), were injected into 6-mm bone defects in an ulnar diaphysis of 2-month old Wistar rats. In situ gel formation was observed intraoperatively. Longitudinal assessment of bone regeneration was done by plain radiograph, micro-CT scan, and histology analysis.

Results: At 12 weeks, more new bone formation was found in the study groups with hydrogel injection compared with the control group, but the difference was not statistically significant. Limited foreign body reaction was observed. The hydrogel degraded into fragments, which were still histologically observed at 12 weeks. Differences in morphometric parameters and histological results could not be demonstrated among the hydrogel groups with or without cell and/or growth factor.

Conclusion: The injectable surfactant-induced Thai silk fibroin/collagen in situ-forming hydrogel has potential for promoting bone formation with good biocompatibility, although evidence of the efficacy in encapsulation of MSCs and growth factor for delivery was not yet clear.

Keywords: Silk, Fibroin, Hydrogel, Collagen, Cell encapsulation, Growth factor carrier, Bone regeneration

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Bone defects are a common and important problem in orthopedic surgery. Standard treatment with bone grafting has considerable shortcomings including donor site morbidity, insufficient graft material for autologous bone grafts, increase the risk of infection, or immunologic reaction to allogenic bone grafts. Synthetic bone substitutes are an available alternative, but they have limited osteogenic efficacy, can result in a local reaction, and raise concern about the time required for transformation and incorporation. Tissue engineering has recently emerged as an option that can potentially overcome the limitations of current treatments. The concept involves three essential elements, cells, scaffolds, and growth factors to regenerate new bone tissue[1].

Hydrogel, a crosslinked hydrophilic polymer network that exhibits a high water content, is generally non-toxic and biocompatible[2,3]. It is used in tissue engineering for encapsulation of living cells as a cell delivery system and serves as a scaffold for tissue regeneration. In addition, it can be delivered into a target site in a minimally invasive manner. However, the gelling process must not cause damage to loaded

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cells that would adversely affect cell survival[4].

Silk fibroin (SF), the structural protein in natural silkworm fiber, is a naturally-derived polymer available in Thailand. Its attributes include good biocompatibility and slow biodegradation. Because it can be processed into hydrogel by chemical and physical methods, it is appropriate for use as an in situ-forming hydrogel for bone tissue engineering. Previous studies by the authors have demonstrated the induction of gelation of SF over a period of 45 to 60 minutes by the addition of surfactants composed of a mixture of oleic acid and poloxamer-188[5].

Collagen, a major protein in the extracellular matrix of connective tissues, which has the properties of biocompatibility and promotion of cell adhesion, has been widely investigated as a biomaterial for tissue engineering. Various designs of scaffolds made from a combination of SF and collagen have been developed for regeneration of many types of tissues. Moreover, enhanced biomineralization has been demonstrated in co-existence of collagen and SF derived polypeptide[6], suggesting that a hydrogel of collagen blended with SF could be a suitable biomaterial for bone tissue engineering. The authors have developed an injectable in situ-forming hydrogel from a combination of collagen and SF induced by a surfactant for bone regeneration. In vitro study of osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells (MSCs) encapsulated in an injectable surfactant-induced Thai SF and collagen hydrogel has been demonstrated to be biocompatible and to have osteo-induction potential (unpublished data). The present study aimed to evaluate the effect of an injectable surfactant-induced Thai Silk fibroin/collagen in situ-forming hydrogel on in vivo bone regeneration in segmental bone defects in an animal model.

Materials and Methods

Study design

Experimental study (animal model).

Animals

Thirty female Wistar rats (two months old) were obtained from the Animal Research Center, Mahidol University, Thailand. The study's animal use protocol was approved by the Ethical Committee of Animal Care and use protocol of Chulalongkorn University.

Material preparation

Cell isolation: MSCs were isolated from the femurs of 3-week old female Wistar rats. The femurs were dissected and cleaned with PBS before being cut at the proximal and distal ends. Bone marrow was flushed out using a 24-gauge needle with 1 ml of alpha-modified eagle medium (α-MEM) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Flushing was repeated until the entire marrow plug was harvested, then the tissue was disrupted by repeated aspiration through a needle. The resulting cell suspension was placed in a 75 cm^2 tissue culture bottle containing 10 ml of medium and cultured in an incubator at 37°C, 5% CO_2. The medium was changed on the fourth day and every three days thereafter[7].

When 80% to 90% confluence had developed, the cells were released from the dish by treatment with trypsin-EDTA and sub-cultured into a new set of culture dishes at a density of 5×10^4 cells/cm^2 as first passage cells. The first passage cells were again sub-cultured before they became confluent and the second passage cells were used for the experiment[7].

Hydrogel preparation and encapsulation of cells and growth factor: *Bombyx mori* Thai silk cocoons (Nangnoi-Srisaket 1) from the Queen Sirikit Sericulture Center, Nakhon Ratchasima Province, Thailand, were used to prepare a sterile aqueous SF solution using a method modified from Ratanavaraporn et al[8] Forty grams of Thai silk cocoons were added to 1 L of boiling 0.02 M sodium carbonate (Na_2CO_3) solution for 20 minutes. The fibers were then rinsed thoroughly with deionized water (DI) to remove the silk glue. The degummed silk fibers were air-dried at room temperature then sterilized by autoclaving at 121°C for 15 minutes. The sterilized fibers were dissolved in 9.3 M lithium bromide (LiBr) solution at 60°C for four hours with a ratio of 4 g:16 ml silk to LiBr. The solution was dialyzed against sterile DI water for 48 hours to remove LiBr. The SF solution obtained was then centrifuged at 9,000 rpm and 4°C for 20 minutes to remove insoluble parts. The final concentration of regenerated Thai SF aqueous solution was about 5.5% to 6.0% by weight. All equipment were sterilized before use and the entire process was performed under sterile conditions. Microbiological attribution tests, including total plate count and total yeast and molds, were used to verify the sterility of the sterile SF solution[5].

Hydrogels were prepared from 4% by weight sterile SF solution and 0.1% by weight sterile collagen solution (Nitta Gelatin Inc., Japan). Gelation was induced by the addition of surfactants made of poloxamer-188: oleic acid in a ratio of 0.07:0.93 by weight. Each sample was then mixed by a vortex mixer

at 2,500 rpm for 90 seconds.

Human platelet rich plasma (PRP) was used as the growth factor in the present study. The PRP was prepared and supplied by National Blood Bank Center, Thai Red Cross Society. Approximately 500 ml of blood from a healthy male donor was collected in combined with 70 ml of anticoagulants and cooled to about 22°C before centrifugation. PRP as an isolated fraction of platelets was collected from the buffy coat after leukocytes had been removed by filtration. One hundred microliters of MSC cell suspension was added and mixed with the vortexed SF/collagen solution to achieve a total volume of 500 ml. The final concentrations of rat MSCs and human PRP were adjusted to 5×10^5 cells/ml and 50 µl/ml, respectively.

The female Wistar rats were divided into five groups of six rats each, a control group and four experimental groups. The four experimental groups were 1) Thai silk fibroin/collagen hydrogel (SF gel), 2) Thai silk fibroin/collagen hydrogel with PRP (SF gel+PRP), 3) Thai silk fibroin/collagen hydrogel with rat MSCs (SF gel+MSC), and 4) Thai silk fibroin/collagen hydrogel with rat MSCs and PRP (SF gel+MSC+PRP). The pre-gelled solution for each group was stored in an insulin syringe before injection into the bone defect.

Surgical procedure and post-operative care

Under general anesthesia, a 6-mm defect was created at the midshaft of each rat's left ulnar bone using a mini-oscillating saw. Anteroposterior and lateral radiograph views were used intraoperatively to confirm the size and location of the bone defect. In the control group, the bone defect was left without any filling before layer-by-layer wound closure. In the four experimental groups, the bone defect was filled with the hydrogel specific for each group using an 18G needle. In situ formation of the hydrogel was observed, after which wound closure was performed layer-by-layer. After the operation, systemic antibiotics and analgesics were administered subcutaneously at regular intervals for five days. The wounds and general health of the animals were observed daily. All animals were cared for following the standard protocol of the Laboratory Animal Center, Faculty of Medicine, Chulalongkorn University.

Evaluation of in vivo bone formation

a) *Plain radiographs*: Plain radiographs of the left forearm in anteroposterior and lateral views were made of all animals at 4, 8, and 12 weeks using the following settings: energy 60 kV, electrical current 30 MA, exposure time 0.1 second, length from radiation source to specimen 10 cm, and X-ray window 100 cm^2.

b) *Micro-computed tomography (micro-CT)*: Three rats from each group were euthanized with 70% CO_2 at four weeks and the remaining three rats in each group were euthanized at 12 weeks. After euthanization, the left forearms were removed for analysis with a micro-CT scanner. Each specimen was held with a 20-mm diameter x 75-mm height holder. The bone defect was scanned in high-resolution mode (energy 70 kV, 114 µA, 8 W, voxel size 15 µm, slice thickness 15 µm, integration time 300 ms). Bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp) of new bone formation were evaluated.

c) *Histology*: Tissue specimens from the bone defect from the three rats in each group euthanized at 4 and at 12 weeks were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in a gradient series of ethanol, embedded in paraffin, and cut through the cross-section axis into 5-mm-thick sections. The sections were then stained with Hematoxylin-Eosin (H&E) and Alizarin Red S.

Statistical analysis

The morphometric parameters from the micro-CT analysis are reported as mean ± standard deviation (SD). Comparison among hydrogels was analyzed using one-way ANOVA followed by post-hoc LSD test. The level of significance was set at p-value smaller than 0.05.

Results

Gross observation

Injection of the hydrogels into the segmental bone defect through a 18G needle immediately after vortex mixing was convenient. Within 20 minutes, the injected pre-gelled solution turned into a soft white gel that was contained in the bone defect with no leakage (Figure 1). Soft tissue edema was generally observed during the first week after the operation. No wound infections occurred in any of the rats. All the animals were in good health for the duration of the experiment.

Radiography and micro-CT imaging

Plain radiographs taken immediately after the operation confirmed the creation of a 6-mm segmental defect in the diaphysis of the ulnar bone. The injected hydrogel was radiolucent. In the control group, the bone defect showed no signs of bone formation in radiographs obtained at any of the time points. In

Table 1. Morphometric parameters of newly formed bone in a segmental bone defect at 4 and 12 weeks

Samples group	BV/TV (%) Mean±SD	Tb.Th (μm) Mean±SD	Tb.N (mm^{-1}) Mean±SD	Tb.Sp (μm) Mean±SD
4 weeks				
Control	0.003±0.002	0.049±0.003	0.719±0.120	1.418±0.245
SF gel	0.002±0.002	0.048±0.012	0.738±0.178	1.401±0.342
SF gel+MSC	0.002±0.002	0.047±0.007	0.505±0.040	1.988±0.157
SF gel+PRP	0.001±0.001	0.054±0.016	0.615±0.198	1.719±0.553
SF gel+MSC+PRP	0.001±0.000	0.045±0.007	0.583±0.137	1.757±0.424
12 weeks				
Control	0.002±0.002	0.146±0.121	0.316±0.153	3.599±1.710
SF gel	0.008±0.004	0.333±0.085	0.324±0.092	3.305±1.092
SF gel+MSC	0.007±0.005	0.252±0.072	0.389±0.192	2.949±1.186
SF gel+PRP	0.009±0.007	0.193±0.224	0.534±0.365	2.526±1.463
SF gel+MSC+PRP	0.010±0.006	0.330±0.127	0.302±0.033	3.381±0.317

BV/TV=bone volume/total volume; Tb.Th=trabecular thickness; Tb.N=trabecular number; Tb.Sp=trabecular spacing

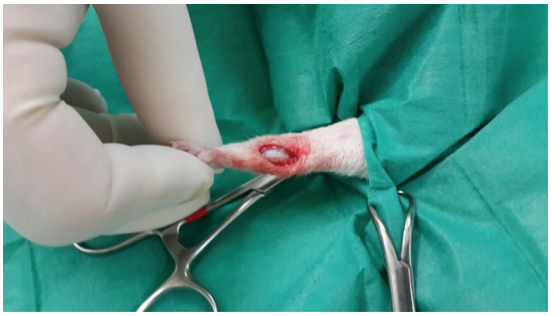

Figure 1. In situ gelling of silk fibroin/collagen hydrogel injected into the segmental bone defect of the ulnar bone of a Wistar rat.

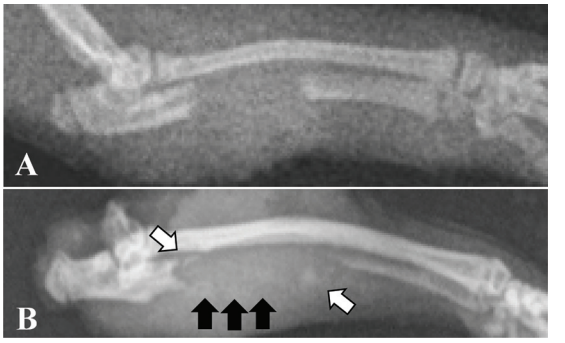

Figure 2. Plain radiographic images at 12 weeks. (A) Control group showed no signs of bone regeneration. (B) Thai silk fibroin/collagen hydrogel with rat MSCs and PRP group showed radiopaque shadow (black arrow) with focal calcification (open arrow) in the bone defect.

the experimental groups, a radiopaque shadow was detected in the bone defect at eight weeks and the shadow density was higher at 12 weeks post-operation although the bone defect was not fully bridged (Figure 2). There were no obvious differences among the four experiment groups.

Micro-CT analysis revealed bone regeneration within the defect site in the four experiment groups that received the hydrogel injection, but only minor bone formation was seen in the control group (Figure 3). Nevertheless, full defect bridging was not reached in any of the groups. Morphometric parameters are shown in Table 1. At 4 and 12 weeks, a statistically significant increase in BV/TV and in Tb.Th was found in the SF gel and SF gel+MSC+PRP groups. There was a trend of increased BV/TV and Tb.Th in the SF+MSC and SF+PRP groups, whereas no increment of BV/TV was found in the control group. At 12 weeks, all four experimental groups showed a superior tendency in BV/TV and Tb.Th compared with control group. No significant differences in outcomes among the groups were detected at either 4 or 12 weeks.

Histological evaluation

Four weeks after the operation, the defect area in the control group showed an empty hole with fibrous encapsulation (Figure 4A). No signs of new bone or vessel formation were detected. In the four experiment groups, the defect areas were filled with gel and appeared as irregularly shaped eosinophilic material surrounded by inflammatory cells and a fibrous capsule (Figure 4B). The number of inflammatory cells was higher in the SF gel+PRP and SF gel+MSC+PRP groups than in the SF gel and SF gel+MSC groups

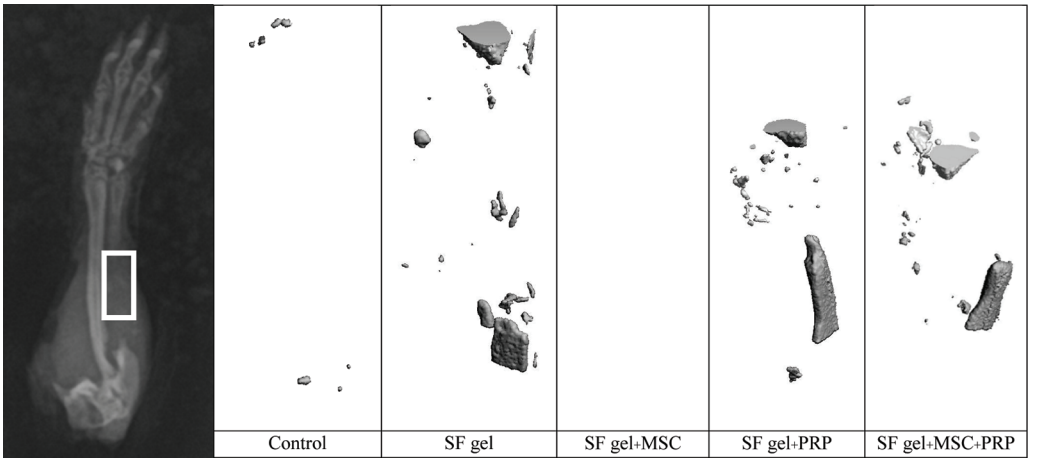

Figure 3. Micro-CT scan images of ulnar bone defects in the control and experimental groups at 12 weeks.

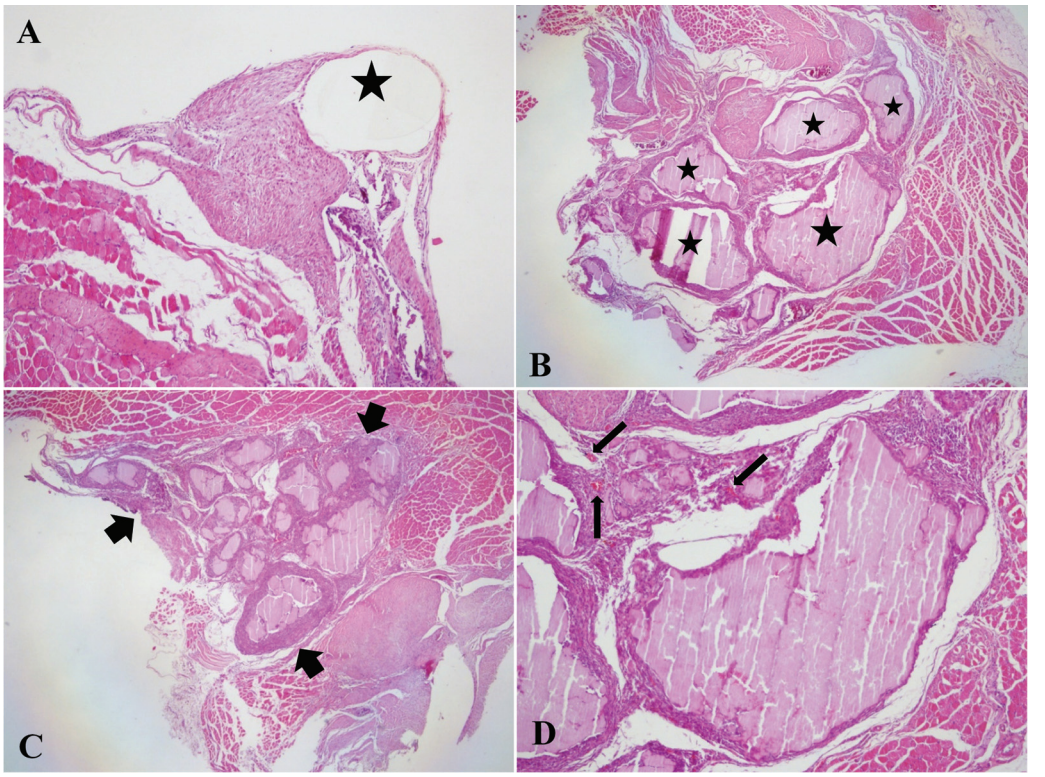

Figure 4. H&E staining of decalcified sections at 4 weeks: (A) An empty defect surrounded by fibrosis (black star) in the control group; (B) Collections of hydrogel appeared as eosinophilic irregularly shaped material surrounded by inflammatory cells (black star) in the Thai silk fibroin/collagen hydrogel group; (C) Higher number of inflammatory cells surrounding gel material (black arrow) in the Thai silk fibroin/collagen hydrogel+PRP group compared with hydrogel without PRP groups, (D) New vessel formation (black arrow) in Thai silk fibroin/collagen hydrogel group shown at higher magnification.

(Figure 4C). Formation of new capillaries was noted in all four experimental groups (Figure 4D). There was only limited new bone formation observed in the SF gel and SF gel+MSC groups.

Twelve weeks after the surgery, the control group still showed no signs of new bone or blood vessel

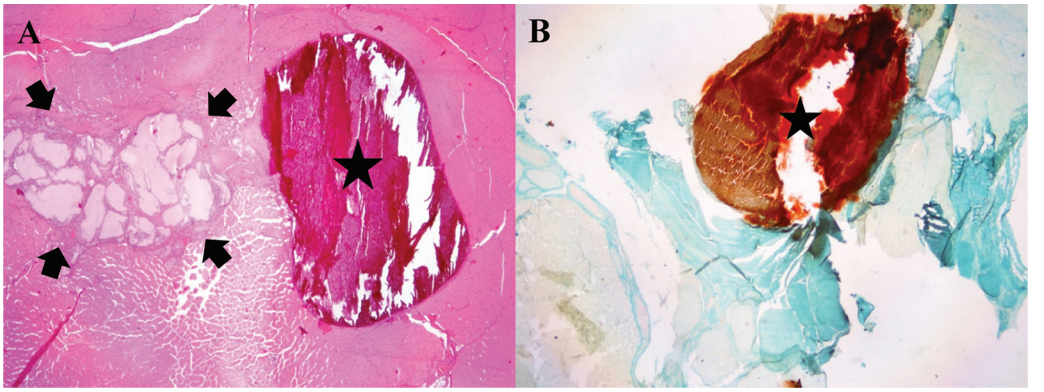

Figure 5. Histological sections at 12 weeks in the Thai silk fibroin/collagen hydrogel with MSCs group: (A) H&E staining showing new bone formation (black star) and residual hydrogel fragments (black arrows) in the Thai silk fibroin/collagen hydrogel with MSCs group; (B) Alizarin Red S staining showing new bone formation (reddish orange material) against a pale green background (black star).

formation. In contrast, there was an increase in newly formed bone at the defect areas of the four experiment groups between weeks 4 and 12 post-implantation. The histologic findings showed relatively similar patterns in the four experimental groups. Fragments of residual nondegraded gel surrounded by fibrosis were observed adjacent to the area of bone formation [*at 12 weeks?] but the amount was substantially decreased from the quantity observed at four weeks. Additionally, there were fewer inflammatory cells at 12 weeks than at four weeks (Figure 5).

Discussion

SF hydrogel has been investigated widely for its physicochemical and biological properties as a potential biomaterial in engineering of various tissues[9]. In bone tissue engineering, SF hydrogel is a suitable biomaterial to be a carrier system for cells and growth factors due to its ability to form a gel in a controlled time with different processing methods[10,11]. Wang et al used sonication to induce SF gelation for cell encapsulation[12]. The capability of sonicated SF hydrogel as a carrier for growth factors (VEGF and BMP) for in vivo bone regeneration has been reported[13,14]. Reports of a combination of SF and other biomaterials aimed at developing blended hydrogel with improved physicochemical and biological properties have been published[15-17]. Hydrogel made by crosslinking SF with collagen, a bioactive extracellular matrix protein, was reported to promote growth without cytotoxicity of vascular smooth muscle cells in vitro[18].

The addition of surfactant was demonstrated to be a useful method for the induction of SF gelation[19,20]. The authors reported SF gelation induced by surfactants comprised of a mixture of oleic acid and poloxamer-188 within 45 to 60 minutes[5], a suitable time for preparation of cell encapsulation, delivery into the target site, and gel setting in situ for treatment of bone defects.

To the best of our knowledge, this is the first study to investigate the efficacy of injectable surfactant-induced in situ formation of silk fibroin/collagen hydrogel for cell and/or growth factor delivery in in vivo bone regeneration.

The results of the present study demonstrate the potential of silk fibroin/collagen hydrogel as a scaffold and carrier for cells and growth factor for bone regeneration in critical size defects in long bones. From micro-CT analysis, all four experiment groups showed an increase in bone volume fraction and bone density of new bone formation at 12 weeks compared to four weeks, while no increase in bone volume fraction was observed in the control group. The morphometric parameters of newly formed bone were comparable among the experimental groups with hydrogel. The histological results were in agreement with the micro-CT results. No new bone or vessel formation was observed in the control group, whereas newly formed bone was found in the four experimental groups with hydrogel. Tissue response to material implantation is a series of events that include blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissues, foreign body reaction, and fibrosis/fibrous capsule formation. These reactions occur very early, typically within two to three weeks after implantation[21]. In the authors experiment, at four

weeks, chronic inflammation was seen and fibrosis was predominant in the four experimental groups. The histological patterns of all four experimental groups were relatively similar with the exception of the inflammatory reaction, which was more noticeable in the hydrogel with PRP added group. Differences in material components that characterize the biocompatibility of the implanted material may be responsible for variations in the inflammatory and wound healing processes[21]. It is noteworthy that the foreign body reaction was generally limited, indicating the biocompatibility of the silk fibroin/collagen hydrogel in vivo. The hydrogel fragments were still noticeable histologically at 12 weeks, but the quantities were much lower than observed at four weeks, results similar to the findings of Fini et al[22] and Zhang et al[14].

Although the beneficial effect of silk fibroin/collagen hydrogel on promotion of bone formation has been demonstrated, comparable morphometric parameters and relatively similar histological patterns observed in the present study indicate that encapsulation of MSCs and growth factor in the hydrogel do not have a positive effect on bone regeneration. That could be the result of using xenogenic PRP from human donors, which may not be compatible with the animal host, thus, causing foreign body reaction instead of enhancing the bone formation, e.g., the histological results showing more inflammatory cells in the hydrogel in the PRP added groups. There have been reports of enhanced bone regeneration using human PRP with and without scaffolds in critical size defects in a rabbit model with no significant inflammatory response observed[23-25], but those studies were conducted by the same group of authors. It is possible that different animal species exhibit different responses. The reason that the advantage from anticipated benefits of encapsulation of MSC in the hydrogel could not be demonstrated is not clear and needs to be investigated further. It is possible that it is due to limited cytocompatibility of the hydrogel with rat MSCs.

Conclusion

The results of using an injectable surfactant-induced Thai silk fibroin/collagen in situ-forming hydrogel as a carrier to encapsulate MSCs and growth factor for bone regeneration of segmental bone defects in long bones in rats indicates that hydrogel of Thai SF blended with collagen promotes bone formation and has good biocompatibility. However, the advantage resulting from encapsulation of MSCs and the growth factor in the hydrogel could not be demonstrated, possibly due to foreign body reaction to the xenogenic PRP and the cytocompatibility of hydrogel with MSCs.

What is already known on this topic?

Hydrogel is a promising biomaterial that can be utilized for cell encapsulation as a cell delivery system and scaffold for tissue regeneration. It can be delivered into target site in a minimally invasive manner. SF is suitable to be developed into in situ-forming hydrogel attributed to its ability to form gel within controlled time by various processing methods e.g., surfactant addition. Induction of gelation by addition of surfactants composed of a mixture of oleic acid and poloxamer-188 in 45 to 60 minutes was demonstrated. Blended hydrogels from combined SF and other biomaterials have been developed to improve physicochemical and biological properties. Enhanced biomineralization was demonstrated in co-existence of collagen and silk fibroin derived polypeptide. SF/collagen crosslinking hydrogel was reported to promote growth without cytotoxicity of vascular smooth muscle cells in vitro.

What this study adds?

To the best of our knowledge, this is the first study to investigate the effect of injectable surfactant-induced in situ formation silk fibroin/collagen hydrogel for cell and/or growth factor delivery on in vivo bone regeneration. The results reported in the present study demonstrated potential of the silk fibroin/collagen hydrogel as a scaffold and carrier for cell and growth factor for bone regeneration in critical size defect in long bone.

Benefits of this study

This study is the first evaluation of the effect of injectable surfactant-induced in situ formation silk fibroin/collagen hydrogel for cell and/or growth factor delivery on in vivo bone regeneration.

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Conflicts of interest

The authors declare no conflict of interest.

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