Treatment of Cartilage Injury in Rat's Knee Joint by Implantation of A 3-D Silk Fibroin Scaffold with Chondrocytes

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Abstract

This study investigated the functional and histological properties of an implantation of silk fibroin/gelatin (SF/G) scaffold enriched with chondrocytes into a rat's knee joint. The animals were divided into 3 groups as 1) a sham group which received an incision on the joint capsule, 2) a blank SF/G group which had a burr hole on the cartilage and transplanted with a blank scaffold, and 3) SF/G+C group which had a burr hole on the cartilage and transplanted with chondrocyte-rich fibroin scaffold. At the 1st, 2nd, 3rd, and 4th week after surgery, each animal was subjected to locomotor activity tests by running on a treadmill for 5 min and followed by climbing on a rotating rod for 5 min, respectively. The results of treadmill test showed that some animals in each group could not walk or run as long duration as 5 min period in the 1st week. However, all of them could run normally on the treadmill in the 2nd week. For the rotarod test, most of the animals in SF/G+C group could not stay on a rotarod for the whole period of 5 min at the 1st, 2nd, 3rd, and 4th week after surgery. In contrast, the results of sham and blank SF/G groups showed significantly longer duration of balancing on the rotarod. Nevertheless, when compared the percent change of durations from the 1st week to the 4th week we found that the SF/G+C group had a greater improvement than the sham and blank SF/G groups. After 1-month locomotor testing, the animals were euthanized and their right knees were kept in 10% formalin for at least 3 weeks, then decalcified in 10% nitric acid before slicing for the histological study. By using H & E staining method, there was a thin layer of new regrowth tissue formed in the damaged bone of blank SF/G group. In the SF/G+C group, however, there were a thicker layer of new regrowth tissue and a piece of scaffold with chondrocytes attached to the damage bone. Altogether, these findings indicate that our silk fibroin/gelatin scaffolds have a high potential for further development to be the biocompatible biomaterial used for the treatment of injury or arthritic conditions of the articular surface.

Keywords: bone regeneration, silk fibroin/gelatin scaffold, physical activity test

Introduction

Osteoarthritis and degenerative articular surface are the most common chronic conditions occurred after a traumatic, inflammation, or arthritic conditions. These pathological joint conditions are frequently found at the weight baring joints, i.e., hip, knee, ankle, and vertebral joints, and increasing the incidence in elderly, obese people and sportsman (Chung & Burdick, 2008; Martel-Pelletier, Boileau, Pelletier, & Roughley, 2008). In 2000, the Ministry of Public Health of Thailand had issued that more than 6 million of Thai people were suffering from these chronic diseases. It was estimated that the number of Thai patients would be doubled in the year 2020. Osteochondral defects are one of the common health problems among the people who are aged, over weight, or having a recent traumatic injury. These cartilage lesions can lead to another complication such as a chronic pain, joint dysfunction, swelling, abnormal joint alignment or posture, and reduction in joint mobility (Buckwalter,

1998). So far, the satisfactory treatment that provides a complete restoration of these articular defects has not been archived. During the past two decades a number of studies have been conducted to investigate the alternative treatments for such lesions. Among current surgical treatments, bone tissue engineering is becoming well recognition as an alternative treatment for restoring articular cartilage injuries (Chapekar, 2000; Mauck et al., 2000).

Since the year 2002, a number of biomaterials used for the fabrication of three-dimensional (3-D) scaffold have been explored and developed by several groups of researchers. The materials used for fabricating biomaterials are variety such as collagen-matrix (Frenkel, Toolan, Menche, Pitman, & Pachence, 1997; Nehrer et al., 1998), hydrogel (Malmonge, Zavaglia, & Belangero, 2000), chitosan-based polysaccharide (Madihally & Matthew, 1999), and silk fibroin (Minoura, Tsukada, & Nagura, 1990). Recently, Tiyaboonchai and co-workers had successfully developed 2 types of 3-D silk fibroin scaffolds such as gelatin-based and collagen-based fibroin scaffolds. These 2 fibroin scaffolds were high porosity and stability, and had a good property for integrated cell seeding (Tiyaboonchai, Chomchalao, Pongcharoen, Sutheerawattananonda, & Sobhon, et al., 2011).

In this study, we employed the tissue engineering by seeding and growing chondrocytes in 3-D silk fibroin/gelatin scaffolds (SF/G) developed at Dr. Tiyaboonchai's lab. The 3-D scaffolds were fabricated from fibroin protein extracted from yellow silk cocoons (*Bombyx mori*) blended with gelatin. These scaffolds could serve as a shelter for chondrocytes to attach and grow. However, the biocompatibility and restoration property of our 3-D SF/G scaffolds on damaged cartilage had not been tested in laboratory animals. The present study, therefore, was designed to address this issue directly by comparing the physical activity of the rats having a sham surgery of the knee joint, a surgery with blank scaffold implantation and a surgery with chondrocyte-riched scaffold implantation, as well as histological study of the articular surface of injured knee joints.

Methods and Materials

Preparation and physical characterization of 3-D silk fibroin/gelatin scaffolds

Silk fibroin (from *Bombyx mori* cocoons) was derived from Institute of Agricultural Technology, Suranaree University of Technology, Nakhonratchasima, Thailand. Type A Gelatin (~ 300 bloom) was purchased from Sigma Chemical (St. Louis, MO, USA). In this study, three dimensional silk fibroin/gelatin (SF/G) scaffolds was fabricated by a freeze-drying technique according to the procedure described in Tiyaboonchai et al. (Tiyaboonchai et al., 2011). Briefly, SF/G scaffold was prepared by adding 4% gelatin aqueous solution into 6% fibroin solution with the blending ratios of fibroin to gelatin of 70:30. Then, the blending solutions were mixed with mild stirring for 20 min. The resulting solutions SF/G was transferred into the molds and kept frozen at -20°C overnight prior to lyophilization for 3 days (PowerDry LL3000, Heto, USA). The dry porous sponges were removed from the molds and treated with methanol for 30 min. Finally, methanol was evaporated at room temperature. The physical properties of SF/G scaffold including morphology, pore size, porosity, swelling and mechanical properties were characterized according to Tiyaboonchai and colleague's method (Tiyaboonchai et al., 2011).

Chondrocyte culture in 3-D silk fibroin/gelatin scaffolds

In this study, rat articular chrondrocytes were isolated from shoulders, hips and knee joint of 4-8 weeks old Spraque Dawley rats with an approval of

Naresuan University Animal Ethic Committee, Phitsanulok, Thailand. The method for chondrocytes isolation was performed according to the procedure of Chomchalao et al. (Chomchalao et al., 2013). The isolated chondrocytes were cultured and expanded in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Rat articular chondrocytes from the second passage (P2) were used for seeding in the scaffolds. The SF/G scaffolds (6 mm diameter, 1 mm thickness) were sterilized by autoclaved and placed in 96-well cell culture plate. Sterile scaffolds were pre-wetted with fresh culture medium and incubated overnight at 37°C under 5% CO_2 conditions. The chondrocytes were trypsinized and seeded on scaffolds at a cell density of 1 x 10^5 cells/scaffold. The constructs were incubated for 2 hours at 37°C to allow cell adhesion to the scaffolds. Then, 150 µl of fresh DMEM medium supplemented 10% fetal bovine serum (FBS), 1% Insulin Tranferrin Selenium (ITS) and 1% penicillin/streptomycin were carefully added into each well. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and the culture medium were replaced every 3 days. After the culture period of 14 days, the cell-seeded scaffolds were collected for animal implantation.

Animals

All the experiments presented herein had been approved by the Board of Naresuan University Animal Care and Use Committee (NUACUC) (Protocol number: NU-AE 58 0819). Fifteen Sprague Dawley (SD) rats aging 2 months, weighing between 200-250 g were purchased from National Laboratory Animal Center (NLAC), Mahidol University, Nakornprathom, Thailand. The animals were housed two-three rats per cage in an animal room of Naresuan University Center of Animal Research (NUCAR) which had received accreditation from AAALAC International. The animal holding room was maintained at a constant temperature of 22±1.0 °C, relative humidity of 45-65%, and 12 hr:12 hr dark-light cycle. These animals had free access to food and water (food pellet formula G82, CPF PCL, Thailand) in their cages.

The animals were randomly divided into 3 groups as: 1) a sham group (n = 5) which received a right knee surgery without an injury on the articular cartilage, 2) a blank silk fibroin/gelatin scaffold (blank SF/G) group (n=5) which received a right knee surgery, drilling a hole on the articular cartilage and implanted a silk fibroin/gelatin scaffold without chondrocytes, and 3) a silk fibroin/gelatin scaffold with chondrocytes (SF/G+C) group (n=5) which received a right knee surgery, drilling a hole on the articular cartilage, and was implanted a chondrocytes-silk fibroin/gelatin scaffold.

Procedures for physical activity testing

Before the knee surgery, each group of the animal was subjected to a daily physical activity training once a day for 1 week. The physical activity training included 1) a running test and 2) a rotarod test (Cechetti et al., 2007; Cha et al., 2007; Heng & de Leon, 2009; Vonsy, Ghandehari, & Dickenson, et al., 2009).

The treadmill test was designed for evaluating the animal's ability to use the affected knee on a moving belt (Figure 1). The treadmill speed was set at a constant of 5 meter per minute and the slope was at 0 degree angle. The total training period was 5 minutes. The cut-off time was determined whenever the animal stop running.

The rotarod test was designed to assess the ability to maintain locomotor equilibrium on a turning rod. In this study, we employed a computer controlled rotarod (TSE RotaRod System, USA) with a rod diameter of 3 cm by setting a constant turning speed of 60 rpm (Bearzatto, Servais, Cheron, & Schiffmann). The duration of staying on a rotating rod was determined from the onset to the point that the animal felt down from the rod. The cut-off time was 5 minutes or whenever the animal felt down from the rotating rod.

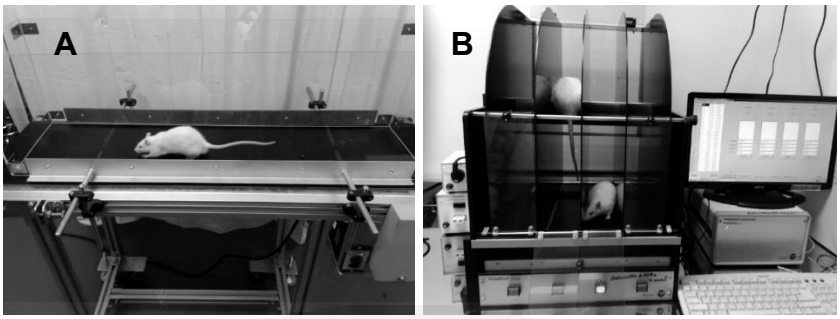

Figure 1 Physical activity tests using a rodent treadmill (A) and a TSE RotaRod System (B). For the treadmill test, the animal was gently placed in the middle of the belt (A). The animal was protected from falling out of the belt by surrounding with the clear plastic side walls (30 cm height). For the rotarod test, the animals were placed on a rotating rod (B) which was set the constant tuning speed of 60 rpm.

Procedure for a knee surgery

The method for a knee surgery was modified from Buvanendran and his co-workers (Buvanendran, Kroin, Kari, & Tuman, 2008). In brief, the animal was deeply anesthetized with 2.0% isoflurane and its right knee was stabilized in a stereotaxic apparatus. After sterilized the skin area over right knee, an incision (10 mm) was made at the lateral aspect of the knee. In sham group, the incision was sutured and then applied with Povidone-iodine (Betadine®). In the control (no treated) group, a burr hole (1.5 mm diameter, 0.5 mm depth) was made on articular surface of the femur, and then a blank fibroin scaffold was placed into the hole before suturing the incision. In the test group, we did the same procedure as the control but applying a fibroblast-fibroin scaffold instead of a blank scaffold. After finishing the surgery, each rat was injected with penicillin 0.04 ml/100 g body weight (BW) at the thigh muscle. After a full recovery from the surgery for 1 week, each animal was subjected to physical activity tests once a week for 4 weeks.

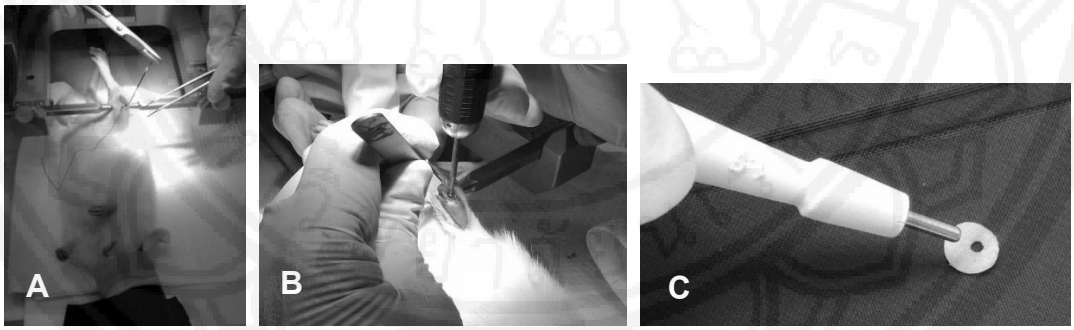

Figure 2 Procedure for knee surgery. A: Under the deep anesthesia with 4% isoflurane, rat's right knee was stabilized by using 2 ear bars of a stereotaxic apparatus. B: a burr hole was made on the femoral condyle using a high-speed drill. C: A piercing device (punch diameter 1.5 mm) and a piece of silk fibroin/gelatin scaffold.

Procedure for histological study

After completing all physical tests, the animal was euthanized with an intraperitoneal injection of 100 mg/kg BW sodium pentobarbital. The right knee of each group was isolated, preserved in 10% formalin for 2 weeks, and then decalcified in 10% nitric acid for a few days. A paraffin block of the tissue specimen was prepared and then sliced into 10 micron thickness. Dying process of these tissue slices was employed by using a Hematoxylin & Eosin method.

Statistical analysis

Data are presented as mean ± standard deviation (S.D.). Multiple comparisons among parametric data were performed using one-way ANOVA followed by post hoc tests for pair-wise comparisons (Dunnett's test). Statistical significant difference was considered at P value <0.05. SigmaStat version 3.0 (Systat Software Inc.) for Microsoft Windows was used for the statistical analyses.

Results

1. Physical properties of the 3-D silk fibroin/gelatin scaffolds

Three dimensional silk fibroin/gelatin (SF/G) scaffolds were constructed using a freeze-drying technique with methanol treatment. Figure 3A showed a gross view of the 3-D SF/G scaffold for chondrocytes culture. Scanning Electron Microscope (SEM) micrographs of SF/G scaffold exhibited a homogeneous porous structure with highly interconnecting pores and smooth surface as shown in Figure 3B. Mean pore size, porosity, water uptake ability and compressive property of SF/G scaffold were 80 ± 28 μm, 61 ± 5 %, 89 ± 3 % and 364 ± 47 kPa, respectively.

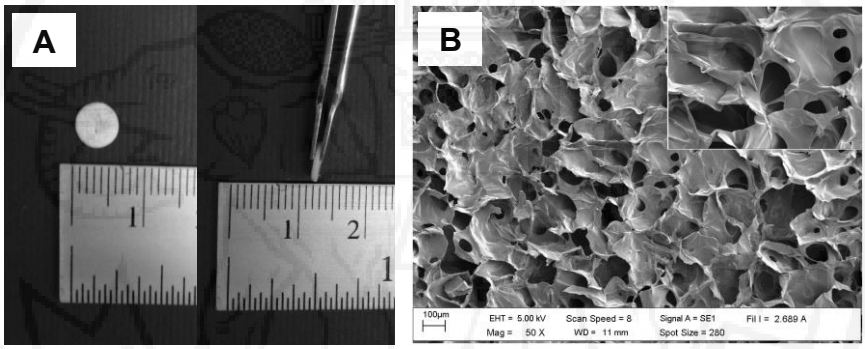

Figure 3 The morphology of SF/G scaffold for chondrocytes cultivation. (A) Gross view of sponge-like structure of SF/G scaffold with 6 mm diameter and 1 mm thickness. (B) SEM image of SF/G scaffold after freeze-drying and methanol treatment.

2. Physical activity tests

After full recovery from the surgery, each group was subjected to treadmill running test. Post surgery for 7 days, both sham and blank SF/G groups showed faster recovery than the test groups (Table 1). The average duration of running of these groups were the same (288 ± 26.8 sec), whereas those of the SF/G+C groups were 200 ± 86.6 sec to 300.0 ± 0.0 sec. Although the running duration on the treadmill of all groups were no significant difference during the 2nd, 3rd and 4th week after the surgery, but if comparing the percent change of the SF/G+C group, they still showed the greater improvement of their physical activities from the 1st week to the 4th week than sham and blank SF groups.

Table 1 Summary of a treadmill test during week 1 to week 4 after surgery.

Duration of treadmill running (sec)					
Treatment	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**% Change**
Sham	288.0 ± 26.8	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0	4.2
blank SF/G	288.0 ± 26.8	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0	4.2
SF/G+C	260.0 ± 34.6*	300.0 ± 0.0	250 ± 86.6	300.0 ± 0.0	15.4

* = $p < 0.05$, one way ANOVA followed by a Dunnett's post hoc test

For the results of rotarod test, both sham and SF-B groups also showed faster recovery than the test group (Table 2). Although some animals of the SF+B group showed a poor ability to maintain on the rotarod after surgery for 1 week, but their physical abilities (% Change) were obviously improved throughout the experiment.

Table 2 Summary of a rotarod test during week 1 to week 4 after surgery.

Duration on rotatod (sec)					
Treatment	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**% Change**
Sham	288 ± 26.8	280.5 ± 33.2	300 ± 0.0	260.1 ± 39.4	-9.7
Blank SF/G	288 ± 26.8	287.5 ± 28.0	275.7 ± 36.1	284 ± 35.8	-1.4
SF/G+C	103.3 ± 110.5*	177.5 ± 56.3*	189.2 ± 126.9	185.0 ± 105.4	79.1

Note: * = $p < 0.05$, one way ANOVA and followed by a Dunnett's post hoc test

3. Histological study

Examples of Hematoxylin & Eosin staining of articular surface of the femoral condyle from sham, blank SF/G, and SF/G+C groups on the 4th week after the surgery were shown in Figures 4. In the sham group, the surgical operation of the joint capsule did not affect the articular surface as shown in Figures 4A and 4B. Normal articular surface of the femoral condyle indicated by smooth and regular surface of the cartilage with good distribution of packed chondrocytes. In blank SF/G and SF/G+C groups, an obvious damage of articular surface could be observed as shown in Figures 4C and 4D.

For the blank SF/G group, the damaged area showed a thin layer of the regrowth tissues covered the underlying bone. In this group of animals, there was no piece of scaffold presented at the damaged area. As shown in Figure 4D, we could observe only a few dispersion of chondrocytes over the affected area (the arrows in Fig. 4D). In contrast, when we observed the articular cartilage of the SF/G+C group, we found that there was a piece of scaffold (SF/G+C) attached at the damaged area (Figures 4E and 4F).

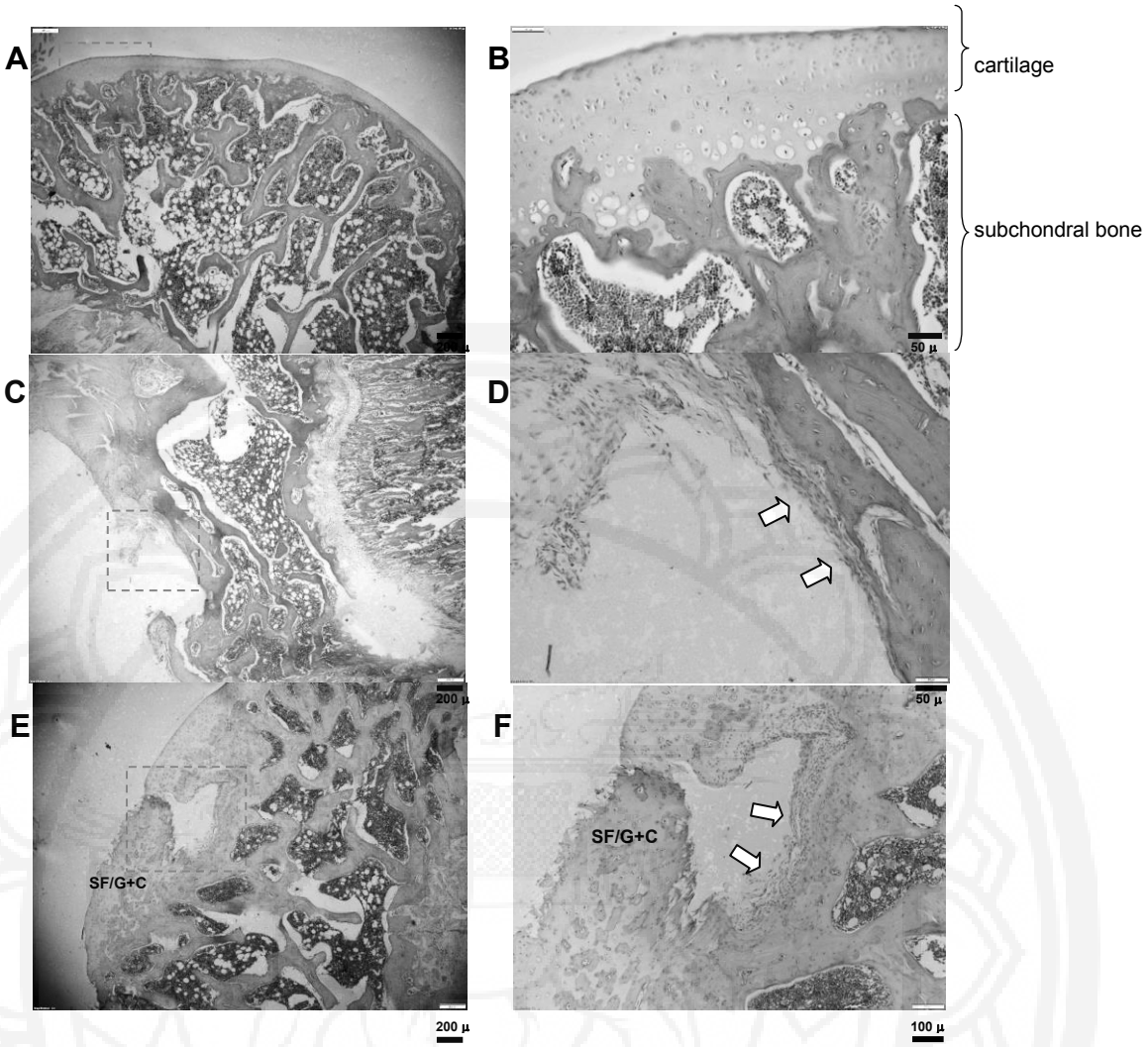

Figure 4 Examples of histologic sections of the femoral condyle obtained from sham group (Figures A and B), blank SF/Ggroup (Figures C and D), and SF/G+C group (Figures E and F) at the 4th week after knee surgery. The small frame in Figures A, C and E indicates the selected area for a higher magnification as shown in Figures B, D and F, respectively. Normal articular surface (Figures A and B) can be observed in sham group. The arrows in Figure D and F indicate the regrowth of chondrogenic tissue over the damaged area (subchondral bone). In Figures D and F, there is a piece of silk fibroin/gelatin scaffold with chondrocytes (SF/G+C) attached at bottom edge of the damaged area. Haematoxylin-Eosin staining magnification: 40x (A, C and E), 100x (F) and 200x (B and D).

Discussion

It is generally accepted that the surgical treatments for osteoarthritis and degenerative articular surface may not totally successful and usually leave some complications after the surgery. Thus, any surgical treatments that promote articular surface restoration would be beneficial to the patients. Among the recent surgical treatments, the implantation of 3-D cell seeded biomaterials is getting more attention among the researchers and clinicians (Chapekar, 2000; Mauck et al., 2000). In this study, silk fibroin/gelatin scaffolds were fabricated from gelatin blended with *Bombyx mori* silk protein which had been proven to be biocompatible and biodegradable (Altman et al., 2003; Vepari & Kaplan, 2007). So far, few studies have been conducted to investigate for general properties and cell viability of different kinds of silk fibroin scaffolds using *in vitro* (Sofia, McCarthy, Gronowicz, & Kaplan, 2001; Kawakami et al., 2011; Talukdar, Nguyen, Chen, Sah, & Kundu,

et al., 2011; Jaipaew, Wangkulangkul, Meesane, Raungrut, & Puttawibul, 2016; Wang et al., 2016) or *in vivo* (Kuboyama et al., 2013) approach. The present study demonstrated good biocompatibility and regeneration of 3D silk fibroin/gelatin scaffolds into the damaged articular surface of laboratory animals. During a 4-week period after the implantation, the treated joint had no signs of swelling or inflammation. It is because silk fibroin is a natural protein fiber extracted from silk of silkworm (*Bombyx mori*) cocoons (Vepari & Kaplan, 2007; Tiyaboonchai et al., 2011) and has been proven for its biocompatibility to both animal and human living tissues (Altman et al., 2003; Lovett et al., 2007).

Even though, the results from physical activity tests indicated that the SF/G+C group did not perform as good as the sham and blank SF/G groups since the 1st week after the surgery, however, these animals still showed their obvious improvements in the 4th week. It is possible that these locomotor improvements may be a result of implanted silk fibroin/gelatin scaffold. Since rats are active and high locomotive animals, therefore, repeated movements of the knee joint may disturb the regeneration process of chondrocytes from the implanted scaffold. This issue may be solved by applying fibrin glues (or gels) between the implanted scaffold and bone defective area (Janmey, Winer, & Weisel, 2009). The factors that might contribute to high variation of the data in the 1st week tests between blank SF/G and SF/G+C groups were possibly due to 1) individual difference in the recovery period and pain level from the surgical wounds (Buvanendran et al., 2008), and 2) a low number of animals in each group. In this study, we did observe that some animals could display spontaneous normal locomotive behavior from day 4 to day 6 after the surgery, whereas a few of them were still limping. Therefore, we suggest for the future study that more number of animals, i.e., at least 10 per group, should be used to reduce the large difference of mean values between these two groups.

Moreover, our histological study showed only partial regeneration of the implanted silk fibroin/gelatin scaffold at the defective cartilage of the SF/G+C group, this was because the bone healing process usually takes from a few months or in some cases might take several months depending on the severity of articular damage (Buvanendran et al., 2008). Nevertheless, the results obtained from this study indicate that chondrocytes-seeded silk fibroin/gelatin scaffold is beneficial to the repair of osteochondral defects. Further studies on an animal model of degenerative joint diseases with longer follow-up periods are needed in order to better understanding of the action of this natural polymer in articular cartilage repair (Li, Chen, Miao, Zheng, & Jin, 2012; Kuboyama et al., 2013).

Conclusion and Suggestion

In summary, the results from this study indicate that SF/G scaffolds are proved to be good shelters for cultured chondrocytes to attach and grow, and good biomaterials for implanting in the injury of articular surface area. The implantation of silk fibroin/gelatin scaffold with chondrocytes can facilitate the healing process of damaged articular cartilage. In addition, histologic examination at the injury site of these groups also showed more regrowth of chondrocytes. Although the physical activities of SF/G+C implanted group were not well correlated with their histological results, we did not observe any sign of inflammation or dysfunction of the affected limb of this group. Our results suggest the potential of clinical application of silk fibroin/gelatin scaffold in the treatment of articular damages. Future studies in different animal models of

abnormal articular conditions are needed to confirm the results of this study.

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