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The Effect of Sericin on Bone Regeneration in a Streptozotocin-Induced Type I Diabetes Animal Model

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Abstract: There is an association between diabetes and impaired bone healing. The purpose of this study was to determine whether sericin had a positive effect on bone regeneration with streptozotocin-induced diabetes in a rat model. Sprague Dawley rats (n = 21) were assigned to one of three groups. A critical-sized bone defect was created on the calvaria. In the sericin group (S group, n = 7), the bone defect was filled with a sericin–gelatin combination, whereas in the gelatin group (G group, n = 7), only gelatin sponge was used. The control group (N group, n = 7) did not receive any graft. New bone formation was evaluated by micro-computerized tomogram and histological analysis. The regenerated bone volume in group S was the highest among the three groups (3.87 $\pm$ 2.51 mm^3), followed by group N (1.71 $\pm$ 1.65 mm^3) and group G (1.24 $\pm$ 1.05 mm^3). The application of sericin in combination with a gelatin sponge enhanced the process of bone regeneration in streptozotocin-induced type I diabetes animal model.

Keywords: sericin; type 1 diabetes; healing; bone graft

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1. Introduction

Diabetes mellitus (DM) is a chronic disease showing an increased sugar level in blood over a prolonged period. Without proper treatment, diabetes can lead to acute complications, such as diabetic ketoacidosis or death due to a hyperosmolar state [1]. Chronic complications are cardiovascular disease, kidney failure, neuropathy, and diabetic feet [2]. There has been a long-standing concern about the association between diabetes and bone health. DM is also associated with bone health. This issue was first suggested nearly a century ago by studying the radiological feature of decelerated bone development and bone atrophy in type 1 diabetes children [3]. The reduction in bone formation during skeletal development is typical in diabetes patients, whereas in adults, hyperglycemia results in the loss of bone by increased bone resorption [4]. Delays in bone union or increases in healing time are features that are most commonly observed when comparing diabetic subjects with matched controls, in clinical studies [5].

In animals with a streptozotocin (STZ)-induced diabetic condition, long-bone fractures show signs of impaired healing [6]. In diabetic animals, femur healing is delayed compared with normo-glycemic controls [7]. There is a double reduction in callus mechanical strength and size in genetic and STZ-induced diabetic animals [7]. Furthermore, the function of osteoblast during fracture healing is decreased in the STZ-induced diabetic condition compared to the control group [6]. Since bone healing is impaired in DM, attempts to improve bone healing have been undertaken. Platelet-rich plasma (PRP), which is thought

to hold high levels of growth factors, has been considered for the treatment of diabetic fractures [8]. The administration of recombinant human bone morphogenetic protein-2 (rhBMP-2) increases the bone formation in diabetic rats via improving angiogenesis [9]. Diabetic animals show low levels of platelet-derived growth factor (PDGF) expression [10]. PDGF is also associated with angiogenesis [10,11]. The administration of PDGF in diabetic animal models shows an increase in cell proliferation and bone formation [11].

Sericin, a protein most commonly biosynthesized by mulberry (*Bombyx mori*) or non-mulberry silkworms (Antheraea mylitta), and also known as silk gum, has the role of enveloping the fibroin [12]. Sericin has a mostly uniform random coil structure with some β-sheet organized structures [13]. The amount of β-sheet structure is increased by stress, such as repeated moisture absorption and mechanical stretching [12,13]. Sericin has many biological properties and has been investigated in tissue engineering [14]. Sericin induces bone-like hydroxyapatite nucleation and sericin-based biomaterials have been tested in bone tissue engineering [15]. High β-sheet content and molecular weight of sericin successfully induces hydroxyapatite nucleation [16]. Since sericin has low-profile mechanical properties, it has not been used for bone tissue engineering without a proper scaffold [17,18].

Based on sericin's property to influence bone regeneration, we hypothesized that the use of sericin would enhance new bone formation in a diabetic animal model. The purpose of this study was to evaluate the effect of sericin on bone regeneration of critical-sized defects in an STZ-induced type 1 diabetic animal model.

2. Materials and Methods

2.1. Sericin Preparation

A silkworm cocoon was harvested from *Bombyx mori*. It was sliced into small pieces and placed in saline. Using a sonicator (40 kHz, 37 °C), a soluble fraction of cocoon was collected for 48 h. The harvested protein solution was filtered using Microcon-30 (Merk Millipore Ltd., Tullagreen, Ireland). The filtered fraction had a high molecular weight (>30 kDa), being identified as sericin by 2D electrophoresis and mass spectrophotometer. The unfiltered fraction (<30 kDa) was a mixture of proteins, such as low molecular sericin fragment, seroin, and protease inhibitors. Sericin of high molecular weight fraction was dried in the form of a film using a dry oven and was stored at room temperature until its use.

2.2. Animal Experiment

The study was approved by the Institutional Animal Care and Use Committee of Gangneung-Wonju National University, Gangneung, Republic of Korea (GWNU-2019-29). Twenty-one Sprague Dawley rats, 8–10 weeks old, were selected for this experiment. At least five days before the injection, the rats were placed in individual cages. On injection day, rats were fasted for 6–8 h before STZ treatment, with a regular water supply. Diabetes was induced by a high-dose (65 mg/kg) STZ injection [19]. The STZ solution was freshly prepared for each injection. STZ was injected intravenously, using a 1 mL syringe and 25 G needle, through the tail vein [20]. The rats were returned to their cages and provided with regular food and 10% sucrose water. On the next day, the regular water supply was resumed. After one week, rats were fasted for 6–8 h to test the blood glucose level from the tail vein, using a blood glucose monitoring system [21]. Animals with blood glucose levels of more than 300 mg/dL were selected for further study. Animals that did not show a sufficiently high blood glucose level received an additional STZ injection.

For surgery, parenteral administration of a mixture was undertaken for the animals. The mixture was Zoletil (Bayer Korea, Seoul, Korea) and Rompun (Bayer Korea). Before incision, the skin on the cranium area of each rat was painted with povidone. After a local anesthesia injection, a vertical incision was made on the middle of cranium. The periosteum was elevated to expose the parietal bones. A full-thickness defect with a size of 8 mm in diameter on each animal calvaria's midline was created using a dental trephine

bur under saline irrigation. Sericin film was melted into sterilized saline and gelatin sponge (Cutanplast Dental®, Uniplex, Sheffield, UK) was soaked in sericin saline solution. The amount of sericin was 0.2 mg/kg for each animal. The sericin group (S group, $n = 7$) received the sericin with gelatin combination as the graft material, whereas the gelatin group (G group, $n = 7$) received only the gelatin sponge. The control group (N group, $n = 7$) had no graft material prior to closure. Closure was performed in layers using 4-0 vicryl. Each rat was injected with gentamicin (0.1 mL/kg; Samu Median, Seoul, Republic of Korea) and tolfenamic acid (0.1 mL/kg; Samyang Anipharm, Seoul, Republic of Korea) intramuscularly from the day before the operation until the third day post-operation. All rats were sacrificed after 8 weeks post-operation, according to the regulations.

2.3. Microcomputerized Tomogram (MicroCT)

After sacrifice, each specimen's calvarial area with a size of 12 mm × 12 mm × 3 mm was harvested for microcomputerized tomogram (microCT) analysis. A SkyScan1173 system (SKYSCAN, Kontich, Belgium) was used to analyze the calvarial specimens. The source voltage was 90 kV and image pixel size was 9.04 μm. Scanned images were then used for three-dimensional data reconstruction using the software available on the device. The size of the primary surgical defect was referenced as the region of interest (ROI).

2.4. Histological Analysis

The decalcification process was performed using 5% nitric acid for 48 h. The specimen was positioned so that the sagittal plane of the defects was shown on the paraffin blocks. The cutting thickness was 5 μm. The slice was put on a slide. These slides were placed into a dry oven at 50 °C for 8 h. Then, the slides were treated by 100% xylene for deparaffin. The subsequent hydration procedure was conducted by the serial application of 100–95% ethanol. After hydration with distilled water, Harris hematoxylin (Sigma Aldrich, St. Louis, MO, USA) was used to stain the sections at room temperature. Specimens were destained by 1% acid alcohol. They were washed in running tap water for 20 min. Next, Eosin Y (Sigma Aldrich, St. Louis, MO, USA) was applied to the tissue sections for 3 min. After serial dehydration with ethanol and clearing with xylene, the tissue sections were fixed by Permount (Sigma Aldrich). A digital camera (DP-72; Olympus, Tokyo, Japan) was used to record the images.

2.5. Statistical Analysis

A one-way analysis of variance test was used for comparison of bone volume among three groups. A post hoc test was performed to compare each group to every other group if statistical significance was found. The least significant difference test was used as the post hoc test. The statistical significance was set at $p < 0.05$. SPSS version 25 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

3. Results

3.1. MicroCT Analysis

The results of the microCT analysis are shown in Table 1 and Figure 1. The largest amount of new bone was found in group S (3.87 ± 2.51 mm^3), followed by group N (1.71 ± 1.65 mm^3) and group G (1.24 ± 1.05 mm^3). The difference in the new bone volume between the groups was statistically significant ($p = 0.035$). In the post hoc test, statistical significance was also confirmed. The differences in the new bone volume were significant between group S and group G ($p = 0.016$) and between group S and group N ($p = 0.042$). However, there was no statistical difference between group G and group N.

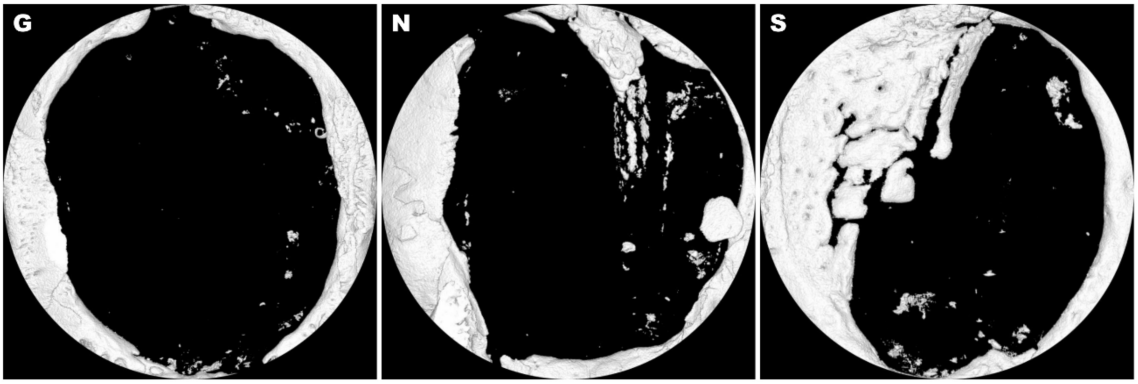

Figure 1. Microcomputerized tomogram (MicroCT) analysis. Scanned images were used for three-dimensional reconstructions. The size of the primary surgical defect was referenced as the region of interest (G: gelatin only group, N: unfilled control group, S: sericin combined with gelatin group).

Table 1. Bone volume in region of interest measured by microCT.

Group	G	N	S
Bone volume (mm^3)	1.24 ± 1.05	1.71 ± 1.65	3.87 ± 2.51 *

* $p < 0.05$, comparison between the S group and the other groups. The values are presented as mean ± SD.

3.2. Histological Analysis

The histomorphometry results are presented in Table 2. The total new bone was similar between group G and group N, at 1.28% ± 1.1% and 1.26% ± 1.05%, respectively. The most important new bone formation was observed in group S, at 8.26% ± 5.43%. The differences between the three groups were statistically significant ($p < 0.05$). The post hoc test revealed a statistical difference between group S and group G ($p < 0.05$). Generally, the formation of the new bony islands in the defect area was more evident in group S. In group G and group N, the bony defects were filled with fibrous tissue or even showed no healing at all (Figure 2).

Table 2. New bone formation in histological analysis.

Group	G	N	S
New Bone formation (%)	1.24 ± 1.10	1.26 ± 1.05	8.26 ± 5.43 *

* $p < 0.05$, comparison between the S group and the other groups. The values are presented as mean ± SD.

4. Discussion

Sericin has demonstrated the ability to enhance bone regeneration in a regular animal model [1]. Silk mat, which is the combination of sericin and fibroin, has increased bone regeneration in clinical trials [22]. In this study, a diabetic animal model was used to determine whether it had a similar effect on a surgically created defect. According to microCT and histological analysis, group S showed more new bone formation compared to group G and group N (Tables 1 and 2).

Studies of sericin have increased in biomedical programs. Stable sericin biomaterials are manufactured by precipitation with ethanol, cross-linking, or incorporation with other polymers [23]. In vitro, mineralized sericin provides better cellular adhesion and spreading than sericin alone [24]. By combining sericin with $CaCl_2$ and Na_2CO_3, spherical particles are formed and mineralized to hydroxyapatite microspheres [25]. Investigation of enhancing osseointegration by coating sericin on titanium surfaces has also been conducted [26]. Sericin has been found to promote the growth and proliferation of mouse osteoblasts [26]. In this study, sericin was extracted by sonication and centrifuging with a filter. Since there was no cross-linking, precipitation with ethanol, or blending with another polymer, the manufacturing procedure was simple.

Several studies have provided evidence of sericin's healing properties, particularly in diabetic models, primarily promoting wound healing. Sericin is used for the treatment of corneal lesions in the type 2 DM model [27]. When 2.4 g/kg of sericin was given to a diabetic animal orally, the treated animals showed reduced blood glucose level and increased neurofilament protein expression in the sciatic nerve [28]. Sericin has a therapeutic effect on dysfunctions of the growth hormone/insulin-like growth factor I axis, which is caused by DM [29]. In this study, a high molecular fraction of sericin was grafted using a gelatin sponge, which had a role of a scaffold for sericin. Compared to the gelatin sponge-only group (G), the sericin combined with gelatin sponge (S) group showed significantly higher new bone formation (Figure 2).

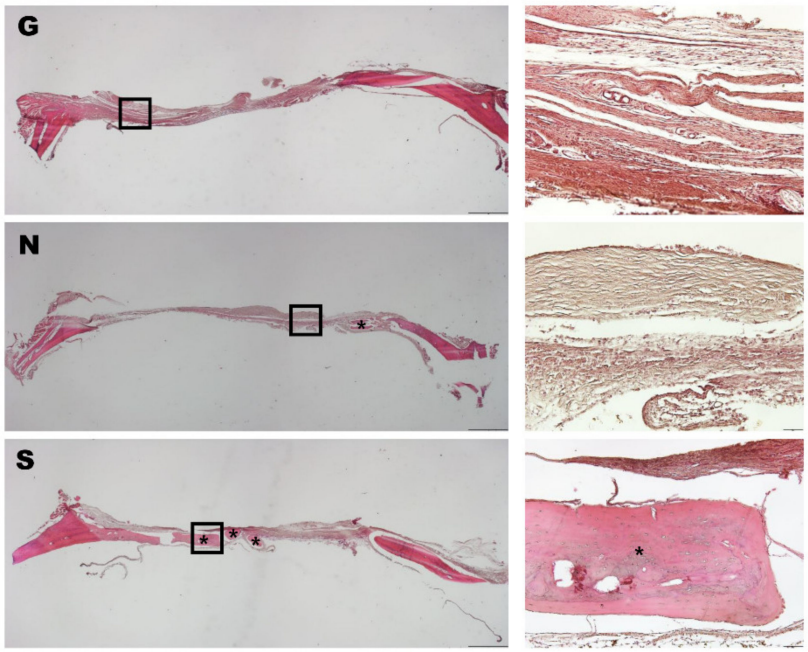

Figure 2. Images of histological analysis. Newly formed bone is represented by the islands marked with "*" located in the defect area (G: gelatin only group, N: unfilled control group, S: sericin combined with gelatin group). Figures in the right column are magnified images of the rectangles.

Bone healing after trauma requires neovascularization and an adequate blood supply [30,31]. Impaired angiogenesis could be the primary cause of delayed fracture healing. Macrophages are essential cells for angiogenesis [32]. Macrophages orchestrate the healing process by phagocytosis and secretion of numerous kinds of cytokines. Macrophage-originated cytokines control wound healing from the inflammatory phase to the remodeling phase [32]. Macrophages can be classified grossly as M1 and M2. M1-type macrophages are predominant in the inflammatory phase, whereas M2-type macrophages constitute the main population in the remodeling phase [33]. Vascular endothelial growth factors (VEGFs) are a key cytokine for angiogenesis and are secreted by M1-type macrophages [33,34]. Sericin is an M1-type macrophage polarizing material and increases VEGFs via the hypoxia-inducible factor-1α mediated pathway [35]. Therefore, the sericin application might help wound healing in diabetic animals via VEGF elevation and subsequent angiogenesis.

Since this study was an observational study of new bone formation, the underlying mechanism of sericin-induced bone formation was under investigation. Although sericin increases several markers associated with new bone formation, such as alkaline phosphatase [25,35], they should be considered to be an end-product of sericin application. Since sericin is a foreign protein for animals, it should show its biological effect via phagocytosis or binding to receptors. If sericin is attached to a certain receptor on the cellular surface, identification of that receptor would be the first step to demonstrate that sericin mediates new bone formation. Toll-like receptor (TLR) is responsible for de-

tecting foreign material [36]. Activation of TLR increases the expression level of bone morphogenic protein (BMP) [37]. The scaffold containing BMP4 increases bone healing in the DM animal model [38]. BMP2 expression in RAW264.7 cells is increased by soluble fraction of cocoon [39]. In this study, high molecular weight fraction only was used as a bone graft. The molecular weight of sericin is different to its extraction technique [12]. As TLR-response is dependent on the size and dosage of foreign material [36], extraction technique may influence on its bone induction ability. This will be a topic of interest in subsequent research.

5. Conclusions

This study demonstrated the effectiveness of sericin in bone regeneration of a calvarial defect of DM animals, providing insight into sericin's potential role as a material in bone tissue engineering. This is the first study using diabetic animals as an experimental group to understand sericin and bone regeneration. Additional studies should focus on the cellular level for a better understanding of the role of sericin in bone formation.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, [KSG], and the first author, [K.D.G.H.], upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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