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The difference in bone morphogenic protein-2 expression level among *Bombyx mori* subspecies

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Abstract

There are several subspecies of *Bombyx mori*, whose silk sericin variants differ. Silk sericin can induce bone morphogenic protein-2 (BMP-2) in macrophages, and silk sericin from different species may have different levels of BMP-2 induction ability. In this study, silk sericin from three *B. mori* subspecies (Baegokjam, Yeonnokjam, and Goldensilk) was prepared. They were administered to RAW264.7 cells and BMP-2 expression level was studied. Bone regeneration was evaluated using a rat calvarial defect model. BMP-2 expression level was the highest in the Baegokjam group. The bone volume in the Baegokjam group was significantly higher than that in the Yeonnokjam group (P = 0.003). In conclusion, sericin from Baegokjam showed higher levels of BMP-2 expression and bone regeneration than those from Yeonnokjam and Goldensilk.

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Introduction

Silk sericin is a protein that encompasses silk fibroin (Jo *et al*., 2020). Silk sericin is approximately 20% of the weight of the silkworm cocoon (Kim *et al*., 2020b). Silk sericin is removed by degumming in the textile industry (Jo *et al*., 2020) and is considered an industrial waste product. Recently, biomedical applications of silk sericin have been intensively studied (Jo *et al*., 2019). Recently, biomedical applications of silk sericin have been intensively studied, such as dressing materials and cosmetics (Kamalathevan *et al*., 2018; Giovannelli *et al*., 2021). Silk sericin has antioxidant and osteoinductive properties (Kim, 2020). High molecular weight of silk sericin induced bone regeneration in rat, whereas low molecular fraction had less effect (Jo *et al*., 2022). These indicated that osteogenic activity is changed depending on the composition of sericin.

The biomedical activity of silk sericin showed different activity according to *Bombyx mori* subspecies (Lee *et al*., 2017). The amino acid sequences in *B. mori* subspecies should be similar. However, each silk sericin has different secondary structure due to differ of location and composition of amino acid.

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According to our recent study, β-sheet content is important in the bone morphogenic protein-2 (BMP-2) induction of macrophages (Jo *et al*., 2022). In addition, some amino acids are acting as ion chelators of magnesium and calcium, which are important for osteogenesis (Wei *et al*., 2022). Therefore, each *B. mori* subspecies may have different biological activities depending on changes in protein structure and chemical composition.

Baegokjam, Yeonnokjam, and Golden silk are Korean silkworms, are subspecies of *B. mori* and their cocoon colors differ. (Lee *et al*., 2017). These differences are due to varying pigments, also the chemical composition might be different depending on the subspecies. The purpose of this study was to investigate the differences in BMP-2 levels after the administration of sericin from each subspecies to RAW264.7 cells. Additionally, differences in bone regeneration were studied in animal models.

Materials and methods

Sericin preparation

Baegokjam, Yeonnokjam, and Goldensilk were prepared by the Industrial Insect and Sericulture Division, National Institute of Agricultural Science, Rural Development Administration. The cocoons were cut into small pieces and 8 L of distilled water was added per 150 g of cocoons. Silk sericin was extracted for 5 h at 121 ℃ using an autoclave (Daihan Scientific, Wonju, Korea). The extracts were then lyophilized for further analysis.

Cell culture and Western blotting

The experimental design of the cellular experiment was described in our previous publication (Jo *et al*., 2021). Sericin from Baegokjam, Yeonnokjam, or Goldensilk were administered to RAW264.7 cells (Korean Cell Line Bank No. 40071). Sericin samples were prepared using the same degumming method. The cells were treated with 1, 5, or 10 μg/mL of each sericin. The cells were collected at 2, 8, and 24 h after treatment and proteins were extracted.

The collected proteins were mixed with sodium dodecyl sulfate buffer. After electrophoresis and transfer to the membrane, blocking was performed using skimmed milk. The membranes were probed with primary antibodies (1:500 dilution). The sources and specifications of the primary antibodies used were as follows: BMP-2 (Santa Cruz Biotech) and β-actin (Santa Cruz Biotech). The blots were visualized and quantified using a ChemiDoc XRS system (Bio-Rad Laboratories).

Animal experiment

The experimental design of the animal experiments was similar to that used in our previous publication (Jo *et al*., 2021). Eight-week-old Sprague-Dawley rats (Samtako Inc., Osan, Korea) were used in this study. All procedures were performed in accordance with the guidelines for laboratory animal care and approved by the Gangneung-Wonju National University for Animal Research (GWNU-2022-7). Sixteen rats (2–3 rats per cage) were housed. Prior to experimentation, acclimation was allowed for one week.

The rats were divided into two groups. Considering the high mortality rate of critical-sized calvarial defect surgery, additional animals were assigned to each group. The graft materials comprised gelatin sponge (Cutanplast Dental®, Uniplex, Sheffield, UK) with sericin from Baegokjam (Group B) and gelatin sponge with sericin from Yeonnokjam (Group Y). The amount of sericin in each graft was ~ 50 μg. The animals in group B (n = 10) were treated with a gelatin sponge containing sericin from Baegokjam. Group Y (n = 6) was treated with a gelatin sponge containing sericin from Yeonnokjam. After administering the anesthetic solution, a mid-sagittal incision was made on the calvaria. A critical-sized defect was prepared in the calvaria using a trephine bur (diameter, 8.0 mm). After placement of the graft, the skin and periosteum were sutured simultaneously using 3-0 black silk. Antibiotics (gentamicin; Daesung, Yiwang, Korea) and analgesics (tolfenamic acid; Samyang Anipharm, Seoul, Korea) were administered postoperatively. The doses of gentamicin and tofenamic acid were 5 and 4 mg/day, respectively. The drugs were administered for 48 h postoperatively. All animals were observed until eight weeks after surgery. After 8 weeks, all rats were sacrificed and specimens from the calvaria were processed for further analysis.

Micro-computerized tomography (mCT) and histological analysis

mCT analysis was performed using a μCT50 (Scanco Medical, Brüttisellen, Switzerland) at the Center for Scientific Instruments, Gangneung-Wonju National University (Gangneung, Korea). The size of the initial surgical defect

Table 1. The results of inorganic components analysis

	Ca	Fe	K	Mg	Na	P	S
Baegokjam	590.14	1.14	363.62	73.68	38.30	20.24	-
Yeonnokjam	518.53	0.91	0	54.11	6.92	16.47	192.95
Goldensilk	489.19	0.68	0	30.73	4.41	20.54	175.95

was referenced to determine the region of interest (ROI). The bone volume (BV) in the ROI was analyzed using the mCT software (CT Analyzer V.1.17.7.2+, Skyscan). The images were analyzed using a lower and an upper grayscale threshold set to 48 and 255, respectively.

Fresh calvarial tissues were fixed in 4% paraformaldehyde at 4°C for 8 h. After washing the fixed samples with tap water, they were placed in a decalcification solution (CAT#: MKCL9701, Sigma-Aldrich). The samples were then placed on a rocking plate for 3–5 d. Decalcification was evaluated using a microtome blade. Decalcified tissues were placed in a tissue processor and embedded in paraffin. To assess the expression of BMP-2, immunohistochemical staining was performed using anti-BMP-2 antibodies (CAT#: sc-137087, Santa Cruz Biotech).

Results

When sericin from group Y was administered to macrophages, BMP-2 expression was observed (Fig. 1a). BMP-2 expression was sericin-dose dependent. However, no protein bands were observed after sericin administration in group G (Fig. 1b). BMP-2 expression level was higher in group B than in group Y (Fig. 1c). BMP-2 expression level in group G was low in this comparative blot. Accordingly, the expression level of BMP-2 was higher in group B than in group G (Fig. 1d and e).

Animal experiments were performed using group B and Y with higher levels of BMP-2 expression than group G. The gelatin sponge incorporated with sericin from Baegokjam (group B) showed higher bone regeneration than that from the Yeonnokjam group (group Y; Fig. 2). The BV was 6.47 ± 3.13 mm^3 for group B and 2.60 ± 0.75 mm^3 for group Y and the differences between the groups were statistically significant (P = 0.003). The histological analysis showed a greater area of bony defects (arrow) in group Y than in group B (Fig. 3).

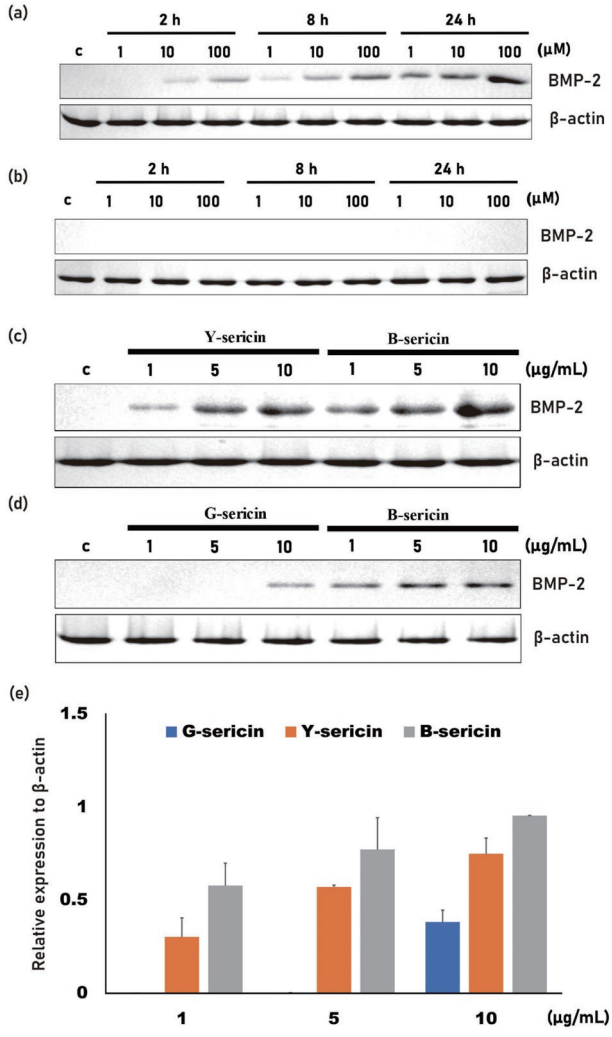

Fig. 1. Western blotting results for each subspecies.

Immunohistochemical analysis showed that BMP-2 expression was higher in group B than in group Y (Fig. 3).

Discussion

In this study, three types of sericin were tested for BMP-2

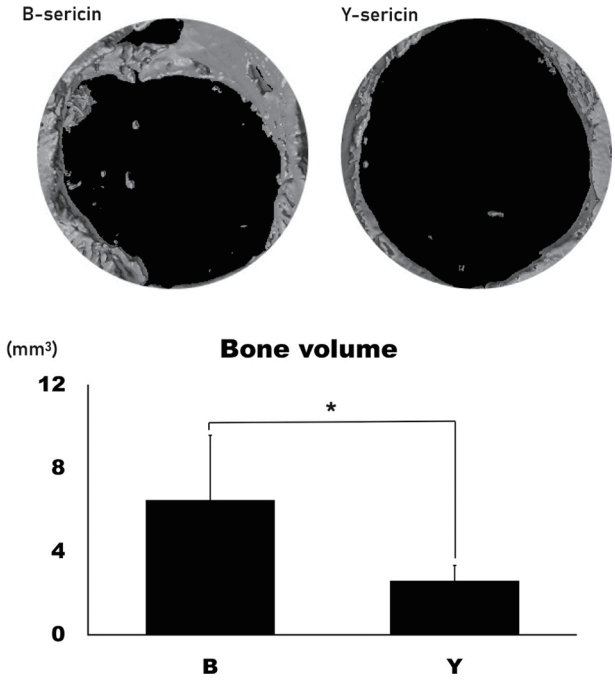

Fig. 2. The micro-CT analysis. Bone volume (BV) was significantly higher in group B compared to that in group Y (P = 0.003).

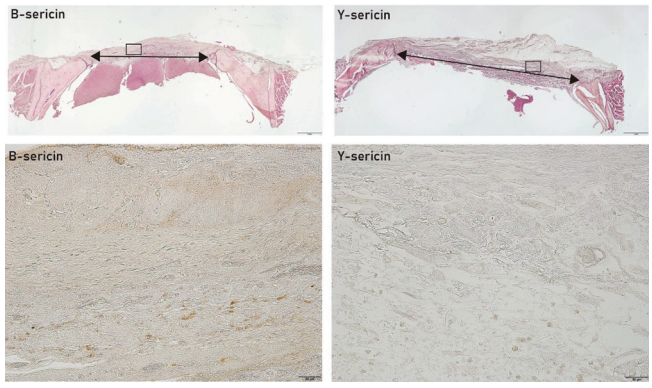

Fig. 3. Histological analysis. The area of bony defect is indicated by the arrow. The defect was larger in group Y compared to that in group B (hematoxylin and eosin stain, original magnification x20). The results of immunohistochemistry were shown in the lower column. The expression level of BMP-2 was more intense in group B than that in group Y. Original magnification for immunohistochemistry was x200 and cropped areas were shown as vacant rectangle on the lower magnification views.

induction in macrophages. The sericin from Baegokjam showed higher levels of BMP-2 expression in macrophages after its administration than those from Yeonnokjam and Goldensilk (Fig. 1). When extracted sericin was incorporated into the gelatin sponge and grafted into the bony defect, BV was significantly higher in the gelatin sponge incorporated with sericin from the Baegokjam group than in the gelatin sponge incorporated with sericin from the Yeonnokjam group (P = 0.003; Fig. 2). Histological analysis demonstrated a higher level of bone regeneration in the gelatin sponge incorporated with sericin from the Baegokjam group (Fig. 3). BMP-2 expression in the tissue was higher in the sericin from the Baegokjam group (Fig. 3).

The silkworm varieties used in this study have different cocoon colors. Baegokjam is the most bred variety in Korea, producing white-colored cocoons with high silk yields (Lee *et al*., 1984). Yeonnokjam is a yellow-green cocoon spinning variety (Kang *et al*., 2011) and Golden silk produces yellow cocoons (Kweon *et al*., 2012). The diverse cocoon colors are due to differences in absorption, metabolism, and secretion in the silkworm's digestive system and silk glands. Therefore, colored cocoons of different varieties may have different chemical compositions (Ji *et al*., 2016).

BMP-2 is a strong osteogenic factor. The combination of BMP-2 with scaffolds has been widely used in bone tissue engineering (Kim *et al*., 2021a). As BMP-2 has a low molecular weight and is highly hydrophilic, many portions of grafted BMP-2 do not remain at the grafted site. Because sustained release is highly important for bone regeneration, a very high dosage of BMP-2 has been used (Zara *et al*., 2011). Excessive BMP-2 may be released in a burst pattern and induce many types of unwanted complications (Simmonds *et al*., 2013). Silk sericin is biocompatible and has a low immunogenicity. The indirect induction of BMP-2 by silk sericin is a physiological method (Kim *et al*., 2020a). To induce BMP-2, silk sericin should activate Toll-like receptors in macrophages in a desirable manner (Jo *et al*., 2021). Binding between the ligand and receptor is important for structural compatibility (Kim *et al*., 2021b). There should be a variant in the amino acid sequence of silk sericin among the different species (Kweon *et al*., 2009). These differences may influence the activation of Toll-like receptors and subsequent BMP-2 expression (Jo *et al*., 2021).

As silk sericin is relatively hydrophilic and easily fragmented in water, proper scaffolds are required for the use of bone grafts (Jo *et al*., 2021). Several types of scaffolds have been tested for silk sericin. The scaffold for silk sericin can be a natural high-molecular-weight protein, such as collagen or gelatin (Vineis *et al*., 2021). Alternatively, synthetic polymers, such as poly lactic acid, poly-caprolactone, and poly (vinyl alcohol) (Zhao *et al*., 2021) may be used. For the same type of scaffold, its

surface texture, porosity, and biodegradability may influence its performance (Geiger *et al*., 2003). As silk sericin has been identified as an emerging active ingredient in bone grafts, different settings of comparative studies should be followed to determine the optimal formula for bone grafting.

In this study, the difference in BMP-2 induction levels was mainly due to differences in subspecies. However, the secondary structure of each sericin extracts has not been examined which affect to binding affinity of osteogenesis receptor. The examination of its binding affinity for the target receptor is further required. Gelatin was used as a scaffold for silk sericin. According to our recent study, acellular collagen showed better performance as a scaffold for silk sericin-based bone grafts (data not shown). If collagen was used for silk sericin, the difference in bone regeneration in the animal model might be less prominent between the groups. The silk sericin release profile from the scaffold in each group may also be interesting. This should be investigated in future studies.

Conclusion

Silk sericin is considered for biomedical applications. In this study, silk sericin from different subspecies showed different levels of BMP-2 induction activity. Among the tested subspecies, sericin from Baegokjam showed the highest BMP-2 induction activity. Accordingly, the sericin from Baegokjam could be a more appropriate source for bone grafts than other sericins.

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References

AOAC (1990) *Official methods of analyses of association of analytical chemist* (15th ed.). Washington DC: AOAC.

Geiger M, Li RH, Friess W (2003) Collagen sponges for bone regeneration with rhBMP-2. Adv Drug Deliv Rev 55(12), 1613–1629.

Ji SD, Kim NS, Kweon H, Choi BH, Kim KY, Koh YH (2016) Nutrition composition differences among steamed and freeze-dried mature silkworm larval powders made from 3 *Bombyx mori* varieties weaving different colored cocoons. Int J Indust Entomol 33(1), 6-14.

Jo YY, Kim DW, Choi JY, Kim SG (2019) 4-Hexylresorcinol and silk sericin increase the expression of vascular endothelial growth factor via different pathways. Sci Rep 9, 3448.

Jo YY, Kweon HY, Oh JH (2020) Sericin for tissue engineering. Appl Sci 10(23), 8457.

Jo YY, Kweon H, Kim DW, Baek K, Chae WS, Kang YJ, *et al*. (2021) Silk sericin application increases bone morphogenic protein-2/4 expression via a toll-like receptor-mediated pathway. Int J Biol Macromol 190, 607-617.

Kang PD, Sung GB, Kim KY, Lee HS, Kim MJ, Ji SD, *et al*. (2011) Breeding of new silkworm strain yeonnokjam. Int J Indust Entomol 23(1), 175-178.

Kamalathevan P, Ooi PS, Loo YL (2018) Silk-based biomaterials in cutaneous wound healing: A systematic review. Adv Skin Wound Care 31(12), 565-573.

Kim SG (2020) Immunomodulation for maxillofacial reconstructive surgery. Maxillofac Plast Reconstr Surg 42(1), 5.

Kim SG, Jo YY, Kweon HY (2020a) Soluble fraction from silk mat induced bone morphogenic protein in RAW264.7 cells. Int J Indust Entomol 41, 51-55.

Kim DW, Jo YY, Kweon HY, Kim SG (2020b) Different level of tumor necrosis factor-a expression after administration of silk sericin fraction in RAW264.7 cells. Int J Indust Entomol 41, 1-5.

Kim BJ, Kim SK, Lee JH (2021a) Bone regeneration of demineralized dentin matrix with platelet-rich fibrin and recombinant human bone morphogenetic protein-2 on the bone defects in rabbit calvaria. Maxillofac Plast Reconstr Surg 43, 34.

Kim SG, Kweon H, Jo YY (2021b) Toll-like receptor and silk sericin for tissue engineering. Int J Indust Entomol 42(1), 1-6.

Kurioka A, Yamazaki M (2002) Purification and identification of flavonoids from the yellow green cocoon shell (Sasamayu) of the silkworm, *Bombyx mori*. Biosci Biotech Biochem 66(6), 1396-1399.

Kweon H, Yeo J, Kim K, Kim Y, Song H, Kim S, *et al*. (2009) Characteristics of silk sericin extracted from sericinjam. Int J Indust Entomol 18(2), 121-124.

Kweon, HY, Lee KG, Park KY, Kang SW, Seok YS (2012). Degumming characteristics and color stability of GoldenSilk cocoon. Int J Indust Entomol 24(1), 1-5.

Lee DY, Cho JM, Yun SM, Hong KS, Ji SD, Son JG, *et al*. (2017) Comparison of silkworm powder from 3 *Bombyx mori* varieties on

alcohol metabolism in rats. Int J Indust Entomol 35(1), 22-29.

Lee SP, Hong KW, Choi SR (1984) Breeding of new spring silkworm variety 'Baegokjam'. Res Rep RDA 26, 58-64.

Schopfer LM, Onder S, Lockridge O (2022) Organophosphorus pesticides promote protein cross-linking. Chem Res Toxicol 35(9), 1570-1578.

Selvakumar G, Lonchin S (2022) Bioactive functional collagen-oxidized pullulan scaffold loaded with polydatin for treating chronic wounds. Biomater Adv 140, 213078.

Simmonds MC, Brown JV, Heirs MK, Higgins JP, Mannion RJ, Rodgers MA, *et al*. (2013) Safety and effectiveness of recombinant human bone morphogenetic protein-2 for spinal fusion: a meta-analysis of individual-participant data. Ann Intern Med 158(12), 877-889.

Vineis C, Cruz Maya I, Mowafi S, Varesano A, Sánchez Ramírez DO, Abou Taleb M, *et al*. (2021) Synergistic effect of sericin and keratin in gelatin based nanofibers for in vitro applications. Int J Biol Macromol 190, 375-381.

Wei X, Zhou W, Tang Z, Wu H, Liu Y, Dong H, *et al*. (2022) Magnesium surface-activated 3D printed porous PEEK scaffolds for *in vivo* osseointegration by promoting angiogenesis and osteogenesis. Bioact Mater 20, 16-28.

Zara JN, Siu RK, Zhang X, Shen J, Ngo R, Lee M, *et al*. (2011) High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. Tissue Eng Part A 17 (9-10), 1389-1399.

Zhao JJ, Liu DC, Yu YH, Tang H (2021) Development of gelatin-silk sericin incorporated with poly(vinyl alcohol) hydrogel-based nanocomposite for articular cartilage defects in rat knee joint repair. J Biomed Nanotechnol 17(2), 242-252.