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Increased Osteoid Formation in Bone Morphogenetic Protein-2 (BMP-2) Loaded Silk-Based Screws in a Rat Model

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Keywords

craniofacial fracture; silk screw; silk-based fixation; bone morphogenetic protein 2; BMP-2; fracture treatment; osteoid formation; resorbable fixation

Introduction

In plastic surgery, osteosynthesis systems are commonly used in craniomaxillofacial procedures including traumatic bone fractures, orthognathic surgery, craniosynostosis and bony reconstruction following head and neck cancer removal. In 2013, over 198,000 maxillofacial surgical procedures performed were reported by the American Society of Plastic Surgeons, reflecting a 150% increase compared to the year 2000.(1) The type of material plays a crucial role in facial fracture management.(2) Conventionally, metallic systems are routinely used for mandible fracture and orthognathic surgery due to robust mechanical properties and ease of implantation/fixation.

Although stainless steel has previously been the material of choice, titanium is currently preferred for internal devices in craniomaxillofacial surgery due to its mechanical strength, osseous integration and superior biocompatibility.(3) However, metallic fixation systems present significant disadvantages including permanence, stress shielding, palpability, loosening and migration, poor bone remodeling, thermal sensitivity, plate exposure, high infection rate and poor wound healing. (4–7) Stress shielding originates from the extreme stiffness of metallic devices resulting in incorrect load transfer to the bone; the elastic modulus of titanium is five times that of bone. This stiffness reduces the density of underlying bone and affects bone remodeling and healing.(4) Furthermore, metallic devices used in pediatric patients may result in implant intracranial migration inciting an

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inflammatory response in the dura and brain tissue (4), inhibition of bony growth when placed across growth sutures and also potential sequelae impacting the mixed dentition of the pediatric mandible.(4, 8, 9). The introduction of resorbable fixation systems was fueled in part by reports of these risks in the pediatric population and has altered the landscape for treatment of conditions requiring osseous fixation in craniomaxillofacial surgery due to its ability to resorb over time, obviating the need for hardware removal due to its degradable nature with controllable rates. (8, 10–17)

Nonetheless, conventional resorbable materials are associated with inflammatory reaction, incomplete integration, formation of sterile sinuses, and may result in incomplete remodeling of the bone exhibiting improper dynamics between device strength retention and wound healing.(11, 12, 18–20) In addition, conventional resorbable plates and screws require laborious implantation techniques because of their fragility. Insertion and implantation requires careful pre-tapping of a pilot hole by the surgeon, as well as pre-shaping of plates to the exact curvature of the complex facial skeleton with the use of heat. (14)

Bone morphogenetic protein-2 (BMP-2) has been studied extensively for its natural role in bone induction as well as its ability to induce bone by external delivery of BMP-2 molecules.(21, 22) Bone will remodel differently in response to mechanical stimuli; the more mechanical load applied to bone, the stronger and denser it will be.(21) This phenomena is essential with regards to resorbable internal fixation since the transfer of stress from device to bone plays a major role in the quality of bone remodeling as the device degrades. Current systems (metallic and resorbable) are associated with poor stress transfer and bone remodeling due to improper degradation dynamics, poor integration and/or differences in mechanical properties.

In this study, we conducted a controlled *in vivo* assessment of a new absorbable osteosynthesis system and propose BMP-2 loaded silk screws would potentially exhibit biocompatibility and promote bone remodeling. Silk fibroin provides an important set of material options for biomaterials and scaffolds in biomedical applications due to its high tensile strength, controllable biodegradability, non-cytotoxicity, low antigenicity and non-inflammatory characteristics.(23) The properties of these new silk-based devices compare favorably with those of current PLGA resorbable fixation systems, however, the advantages of the silk systems include an easier implantation technique, conformal fit to the repair site, autoclave sterilization and minimal inflammatory response. Previous work from our group demonstrated the initial feasibility of silk hardware production.(24) The proposed system of orthopedic hardware will initially target non-load bearing applications with a primary focus on the craniomaxillofacial complex.

Materials and Methods

Production of BMP-2 loaded silk screws

Silk is a fibrous protein polymer that is spun by Lepidoptera larvae such as spiders, silkworms and flies.(23) The most widely studied and used silk is that of *Bombyx mori* silk worms and is composed of two proteins: silk fibroin and sericin. Sericin is a globular protein

that acts as glue for the fibroin fibers and provides structure and support for the silk cocoon. However, sericin has been proven immunogenic and for this reason is removed from the silk fibroin before processing in biomedical applications.(23, 25) The longer the boil time, the longer the silk is exposed to the heat and salt resulting in more sericin removal and a higher degree of silk degradation. The primary silk format in the present work is a dense, non-porous scaffold prepared with the organic solvent 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP), which is a highly effective stabilizer of the silk peptides and promotes strength. The high strength with ability to degrade renders HFIP silk solutions a promising format for silk orthopedic hardware. There is usually a trade-off between the strength and degradation.(23, 26) Silk solution was prepared under established silk processing and purification protocols (Figure 1). (26) Silk screw blanks were prepared using wax molds from MachinableWax.com (Traverse City, MI, USA) and machined to molds (Figure 2). The silk blanks were machined down to the desired outer diameter or head diameter and bone screw threads were created using an external single point cutting tool. Silk pins were machined to 1.5 mm diameter and 3.0 mm length.

Functionalization with BMP-2

Pins were placed in a solution of BMP-2 for 4 days at -4°C in microtubes to load the pins with BMP-2. To quantify BMP-2 release in an aqueous environment in vitro assessment was performed on 3 screws. Once dry, the BMP-2 loaded silk pins were placed in 150 μl of phosphate buffered saline (PBS) for release. PBS was sampled and replaced at 1, 2, 4, 6 and 24 hour, 2 and 5 days. Samples were then tested for BMP-2 using a BMP-2 ELISA kit to manufacturer's instructions. Release data can be seen in Table 1 and is represented as quantity of BMP-2 released as percent release.

Experimental Protocol

A total of 15 female, 250 gram, Sprague Dawley rats were used for all experiments. The experimental protocol was approved by the animal care and use committee at Tufts University. After inhalational induction of anesthesia with isoflurane, a 10mm longitudinal incision was made along the hind limb. The vastus lateralis and biceps femoris were then separated along the length of the femur. The distal end of the femur was exposed and a 1.5mm diameter, 3mm length uni-cortical hole was drilled with a Synthes Electric Pen Drive (DePuy Synthes Inc., West Chester, PA, USA). Screws were inserted with the use of a standard flat head screwdriver by self-tapping, similarly to conventional metal systems (Figure 3). Finally, the incision was closed with 5-0 Vicryl sutures in a simple interrupted pattern. All surgeries were performed by an experienced surgeon.

Animals were sacrificed at 1 month, 3 month and 6 month time points. 5 Animals were used for each time point. For 4 of the 5 rats per group, a BMP-2 loaded silk screw was placed in the left hind limb and a control silk screw, without BMP-2 load, was placed in the right hind limb. In the 5th animal per group, a hole was placed with no screw in the left hind limb and a stainless steel screw was placed in the right hind limb.

Histopathological analysis

The femora were explanted and dissected for histological evaluation. Samples were decalcified and fixed in Surgipath®. After 5–7 days tissue samples were fixed in paraffin and sectioned with a thickness of 5 µm using a microtome. Finally, sections were stained with Masson-Goldner trichrome (MTC) and hematoxylin and eosin (H&E) stains. Sections were evaluated on the presence of osteoblast/osteoclast cells, deposition of collagen and osteoid, inflammatory response, and the level of cell infiltration. Cell infiltration and osteoid deposition are important observations for the evaluation and validation of osteoinduction by BMP-2. In the remodeling process of bone, the BMP family is known to promote cell proliferation, to attract osteoblasts/osteoclasts and to generate woven, mineralized collagen deposition.(21)

Results

All animals tolerated the procedure well and no complications occurred during implantation of the screws. In all 15 animals, rats were mobile on all four legs immediately after the effects of anesthesia diminished post-operatively. None of the animals appeared to show signs of pain during examination for 3–4 days post-surgery or have issues with infection or wound dehiscence. Intraoperatively, screws came in contact with blood and other bodily fluids but were able to maintain integrity for insertion. All samples were explanted at the designated time points and sent for histological evaluation. No animals died or had to be terminated during the 6 month study period. In vitro studies demonstrated an initial peak in BMP-2 release (range 11.6% – 27.4%) within the first 2 hours after contact with PBS. Table 1 shows an initial peak in the first 2 hours after contact with fluids with a steady release of 0.1–0.4% per day in the following days.

Histopathological analysis

Figure 4 UX and 5UX show histological cross sections of silk screws and surrounding tissue after 1 month. Screw placement can be clearly observed by the screw void. The delineation between dark blue and light blue represents the transition of pre-existing bone to newly formed unorganized bony matrix deposition, respectively. The width of the pilot hole appears wider at the top of the screw most likely due to drilling and insertion. It is visible in the MTC stains that good collagen deposition has been induced between the screw and edge of the original pilot hole. Woven, mineralized collagen around the periphery of the screw appears to be greatest and most organized in the BMP-2 loaded samples. Collagen has also been induced around the top of the screw head, which was only seen in the BMP-2 loaded samples. A mixture of granulation tissue and early cartilage formation can be observed indicating a normal bone healing process. In Figure 5U and 5X, osteoclasts and osteoblasts can be seen along the periphery of the silk screws as well as within the osteoid/collagen matrix. Cell density, organization of new collagen deposition and quantity of new collagen deposition appears highest in the BMP-2 loaded sample.

The 3 month histological cross sections further support the results observed in 1 month explants. It can be seen that at 3 months the BMP-2 loaded silk screw (Figure 4V and 4Y) is surrounded by a thick layer of new woven, mineralized collagen. There is less granulation

tissue and more organized bone formation. In comparison to the non-loaded silk screws (Figure 4Y and 5Y), the BMP-2 sample has a higher degree of collagen deposition, the deposition is more ordered, and the new collagen has a darker color signifying it is further in the development process to mineralized bone. No inflammation or negative host response was observed in any sample. Little bits of pink screw material are noted within big multinucleated cells, indicating osteoclasts consuming the screw. The lack of any negative response supports the safety and biocompatibility of the silk hardware.

By 6 months, more silk screw material has been absorbed and cellular organization has improved further. In both examples (Figs. 4, right, and 5, right) the bone appears to form lamellar bone, and predominantly osteoblasts and osteoclasts are seen. Osteoclasts are seemingly consuming parts of haphazard bone. Bony callus formation which was seen at 3 months has been replaced by more organized bone; these findings support a normal bone remodeling process. There was no scar formation. Also, at this junction there was a virtually complete absence of inflammation in all samples. Increased bone remodeling was noted in the BMP-2 loaded silk screw sections at 3 and 6 months as compared to non-loaded screws, which is best observed in the osteoclastic activity immediately surrounding the screws in the MTC stain (Fig. 4, right). None of the explanted samples showed a final stage of bone healing.

Discussion

Metallic fixation systems have been widely used due to their strong mechanical properties, good biocompatibility, and simple implantation technique. However, growth alteration due to rigid nature of the metallic device may occur. (27) Subsequent loosening of the metallic implants may cause corrosion exciting an inflammatory response potentially resulting in implant extrusion. Furthermore, visibility, palpability, poor bone remodeling, possible need of second surgery removal and cold sensitivity are other potential problems of metallic fixation systems. Fracture stabilization by rigid internal fixation devices is only necessary until fracture healing is complete, after which the devices is rendered useless(28). Development and advances in the medical use of biodegradable materials have led to the development of bioabsorbable orthopedic hardware systems to address shortcomings of metallic systems.

Resorbable systems have unique advantages in treating fractures located in areas such as the craniomaxillofacial skeleton. First of all, the relative rarity of solid compact bone in the midfacial area is an important factor. Rigid fixation with metallic devices can accelerate stress shielding of underlying cancellous bone. Also, the facial area has thin coverage over the bone leading to a high possibility of plate visibility or palpability, which may be particularly important in cosmetic outcome. Repeated procedures for fixation device removal can cause soft tissue deformity and unfavorable scar formation. The use of biodegradable hardware has been supported by clinical studies demonstrating sufficient strength for fixation of non-load bearing fractures which can be achieved with resorbable systems that do not require removal and provide a more gradual transfer of stress from device to bone allowing for improved bone remodeling(5, 7, 8, 10, 14, 29–35) The use of resorbable hardware obviates the need for second surgery removal and limits complications

such as thermal sensitivity, issues with radiological imaging and stress shielding due to extreme stiffness of metals. (36, 37)

Various resorbable systems have been available for many years but their clinical applications have been limited particularly by potential infection rate and implantation technique. The most commonly used biomaterials as a basis for resorbable systems include polyesters such as polylactic acid (PLA), polyglycolic acid (PGA) and poly-lactic-co-glycolic acid (PLGA) (36, 37) and degrade in about 2–6 years, 1 year and >1 year, respectively. Their application is primarily limited to specific applications such as pediatric craniofacial reconstruction(8), fixation of metacarpal fractures(13–15) and long-term soft-tissue moulding.(38) Swelling and inflammation have remained an issue when PGA is used, with reported rates up to 60% (5, 36, 37, 39). PLGA is promoted to improve bone remodeling through absorption and degradation, however, results have shown incomplete remodeling and that the resorption is not osteoinductive. (40, 41). Park et al studied the effectiveness of resorbable fixation device in the treatment of zygomaticomaxillary fractures and found complication rates of 7% with resorbable skeletal fixation systems and 4% with metal plate-and-screw systems. (20) Despite histological studies on resorbable systems focusing on an increased inflammatory response, multiple clinical outcome studies present potential benefits of resorbable devices. (8, 10–15, 42–45) Van Bakelen et al reported that both metallic and resorbable systems had good mandibular function and were free of pain in their two-year multi-center randomized controlled trial. (44) Furthermore, a study done by Fedorowicz et al reported no statistically significant difference in postoperative discomfort, level of patient satisfaction, plate exposure or infection for plate and screw fixation using either titanium or resorbable devices in orthognathic surgery. (42) This was supported by Yang et al, who conducted a meta-analysis to compare complications between metallic and resorbable fixation in maxillofacial surgery and demonstrated absorbable fixation systems to have a similar favorable safety profile as titanium fixation during Le Fort I, bimaxillary operation, and bilateral sagittal split ramus osteotomy procedures conducted. (45) Regarding resorbable plates and screws, infection rate and difficulty of implantation have not limited the clinical applications of resorbable systems. However, these well-known drawbacks have undoubtedly influenced surgeons' willingness to use them particularly in the adult patient when compared to conventional metallic alloy.

Silk has been widely used since its first use for sutures decades ago and has shown excellent properties such as biocompatibility, biodegradation, and non-toxicity.(46) It presents a promising candidate material for biomedical applications due to its numerous beneficial properties including excellent biocompatibility, good oxygen and water vapor permeability, and biodegradability.(23) The silk fibroin matrix is known to support the attachment, growth, and differentiation of adult human, progenitor bone marrow stromal cells and to enhance bone regeneration.(23, 47) Its physical and chemical properties can be easily modified to achieve desirable geometries, size and degradation characteristics allowing for flexibility in future designs.(46) It is degradable in vivo with controllable rates from days to years and maintains structural stability to >170°C, are stable at extremes of pH and can be autoclaved for sterilization; all features that enhance potential utility in new medical devices when added to the mechanical features and biocompatibility.(23, 48) Lee et al. report on the use of a silk fibroin – bacterial cellulose composite plate to augment new bone formation in

zygomatic arch defects.(49) Moreover, silk has the capability of being functionalized with bioactive compounds including BMP-2 for delivery in vivo.(50–57).

In the present work, silk orthopedic hardware was functionalized with BMP-2 through absorption of BMP-2 solution based on loading quantities in previously published work.(51, 52) At 1, 3 and 6 months, histological evidence supported the release and activity of BMP-2. At 1 month the BMP-2 loaded implant histological sections showed the greatest quantity of new collagen deposition with high cell density consisting of osteoclasts and osteoblasts compared to non-loaded samples. The deposition of collagen and osteoids extended even over the head of the screw. No inflammation or negative side effects were noticed in H&E and MTC stained slides. In our 1 month histopathological study we could confirm initiation of the remodeling process by observing the recruitment of multinucleated, macrophage-like cells, possibly representing osteoclasts and early osteoblast deposition of osteoid. This finding is consistent with other studies which demonstrated that silk fibroin induces bone tissue growth of osteoblasts or stem cells in vitro (23, 58, 59). Kim et al(47) reported that silk fibroin nanofiber membranes had good biocompatibility and enhanced bone regeneration in a rabbit calvarial model, suggesting that the silk fibroin could be useful in guided bone regeneration. The results we found after 3 and 6 months supported our first histopathological observations at 1 month. Also, consistent with other studies previously reported, no pathological inflammation was demonstrated in any of the sections.(60) Moreover, our BMP-loaded samples continued to show greater recruitment of osteoclasts and osteoblasts around the perimeter of the BMP-loaded screws.

Even though resorbable systems possess several benefits over metallic systems, wide clinical application is constrained mainly by their laborious implantation technique. However, silk screws can be implanted with an identical technique to current metallic systems, without the need for bone pre-tapping. The ability to be functionalized with and successfully release BMP-2 during in vivo experiments supports the feasibility of producing osteoinductive fixation devices and demonstrate potential for the loading and release of other molecules.

Our study was subject to several limitations. In order to quantitatively measure the effect of BMP-2, a method that observes mineralization and/or bone formation such as a von Kossa stain would need to be utilized in conjunction with X-ray diffraction. (61) Also, despite successful demonstration of in vitro release of BMP-2, determination of loading quantity will need to be further investigated. A major issue in the production process of silk as orthopedic hardware was that a large quantity of material was wasted due to bubble formation during the fabrication process. Lastly, as previously stated, resorbable devices currently used are for non-load bearing craniomaxillofacial applications for which the weaker mechanical properties are sufficient.

With these successful outcomes with silk screws, further enhancements can be achieved by controlling mechanical properties and incorporating various bioactive compounds. Potentially, antibiotics can be loaded to minimize infections and inflammation, or proteases could be added to facilitate more rapid degradation. (23, 48, 51–54, 62–65) Silk-based fixation devices can be tuned to degrade in a shorter time frame to allow for dynamic transfer of the load back to the healing bone. Future work may also focus on percent of

retained screw, replacement with bone and fusion with native bone, as well as inclusion of a PLGA control group to compare biocompatibility between silk and PLGA to further enhance the quality of data reported.

Conclusion

The BMP-2 loaded silk-based fixation device in this study supported previous results of safety and biocompatibility in non-loaded silk screws. Notably, BMP-2 appeared to be successfully released resulting in increased collagen deposition and cell density. The release of BMP-2 would allow silk hardware to facilitate osteoinduction and result in more complete bone remodeling. Functionalization is not limited to BMP-2 but could potentially be an approach utilized to minimize infection rate and other side effects by the incorporation of antibiotics and other therapeutic compounds.

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Figure 1.

Silk cocoons are fragmented and boiled to remove sericin due to its immunogenic character. The silk is then rinsed, dried and dissolved with heat in a solution of lithium bromide (LiBr). Next, the LiBr salt is removed through dialysis and combined with 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP). This combination is injected into a mold and submerged in methanol to crosslink the silk fibers. Subsequently, the silk blanks are removed and allowed to dry, after which the blanks are machined into screws.

Figure 2.
Example of silk screws (1.5 × 3.0 mm)

Figure 3.
Intraoperative placement of a BMP-2 loaded resorbable silk screw

Figure 4.

Masson-Goldner Trichrome stains. Histological cross sections of bone including BMP-2 loaded (U, V, W) and non-loaded (X, Y, Z) silk screws at 1, 3 and 6 months. The void from silk screws appears white, indicating the silk became removed during sectioning. [C] osteoclasts/osteoblasts/cells, [S] screw placement, [B] bone.

Figure 5. Hematoxylin and Eosin stains. Histological cross sections of bone including BMP-2 loaded (U, V, W) and non-loaded (X, Y, Z) silk screws at 1, 3 and 6 months. The void from silk screws appears white, indicating the silk became removed during sectioning. [C] osteoclasts/osteoblasts/cells, [S] screw placement, [B] bone.

Table 1.

Percentage (%) release of BMP-2

	1hr	2hr	4hr	6hr	1D	2D	5D	Total (%)
Screw 1	17,507	19,710	0,159	0,131	0,133	0,121	0,110	37,871
Screw 2	27,410	11,601	0,141	0,325	0,244	0,140	0,281	40,142
Screw 3	25,290	27,165	0,290	0,268	0,365	0,107	0,147	53,632

hr = hour; D = day(s)