Use of Silk Scaffolding to Modulate the Interface Between Skin and Percutaneous Implants of Osseointegrated Prostheses for Patients With Limb Amputations

Student Role: The student (Jon Florance) participated in developing the study design, assisted with specimen harvesting and mechanical testing, wrote the final manuscript, and presented the results at the Harvard Orthopaedic Trauma Research Day.

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Background:

Limb loss has devastating physical and psychological impacts. In addition to disfigurement, decreased functionality can challenge independence and reinforce negative psychological effects. This loss of function can be especially disruptive for young healthy patients, including the over 1,600 combat related amputations of service members from the Global War on Terror and thousands of other amputations in the United States resulting from trauma, who immediately transition from an active lifestyle to decades of impairment.[i] This active cohort is frequently ill-suited for traditional socket prostheses which place considerable pressure and friction on the skin of the residual limb, resulting in open sores, pain, and poor proprioception that limit overall mobility.[ii,,iiiiv] In contrast, these patients may be ideal candidates for surgically implanted osseointegrated prostheses which provide better quality of life, proprioception, and mobility.[v,,vivii]

Percutaneous osseointegrated prostheses install a post directly into the medullary cavity that protrudes through the skin with an abutment that accommodates the prosthetic attachment. While recent research has demonstrated some success in limiting the traditionally high infection rate, no technique has been able to produce an efficacious seal between skin and implant that could effectively normalize infection risk in the long term.[viii] The leading purported contributors for this faulty seal are differences in mechanical properties between the elastic skin and rigid metal implant combined with the inability of skin cells to adhere to the implant surface.[ix] The result is a downgrowth of the epithelium along the implant producing a sinus tract capable of seeding the bone with surface bacteria.[x,xi] Ideally, an effective seal would inhibit epithelial downgrowth and maximize the underlying soft tissue integration with the implant to prevent the passage of pathogens through the integumentary system.[xii]

This initial study was conducted to investigate the use of silk proteins as a subcutaneous scaffolding material to address the skin implant seal for percutaneous osseointegrated prostheses. Silk was chosen due to its biocompatibility and degradability, strength and longevity, and its ability to slowly release embedded compounds such as growth factors or antibiotics. The use of silk scaffolds in burn wounds has already demonstrated how leveraging these properties can result in decreased infection rates and increased vascularization, collagen deposition, and tissue organization.[xiii,xiv] In this study, we hypothesized that subcutaneous silk scaffold implants would bridge the difference in mechanical properties of the flexible skin and rigid post while promoting fibrosis within the subcutaneous tissue; the former effect would decrease shearing forces surrounding the implant abutment and the latter would increase soft tissue integration with the body of the implant, both working synergistically to combat the downgrowth phenomenon.

Study objective: To change the mechanical and histological properties of the skin using silk implants in a rat model.

Study Design:
Six adult male Sprague-Dawley rats (aged 13 weeks) were subjected to surgery in the form of subcutaneous insertion of a silk implant. Ribbon shaped silk implants were used that measured 50mm in length, 5mm in width, and 1mm in thickness with 2mm long projections angled at 45^{o} from one surface. Silk implants were inserted longitudinally underneath the right side of the dorsal skin region between the shoulder and pelvis girdles and 15 to 20 mm lateral to the midline. Animals were euthanized 10 weeks after the surgery and the dorsal skin on both sides were harvested. Harvested skin from the right side was used as the intervention specimens while skin from the left side was used as the control. All specimens were subjected to mechanical testing and histology studies.

Surgical Procedure:
General anesthesia was induced using Isoflurane at 5% in an induction chamber then maintained at 2.5% via a nose cone. Following anesthesia, animals were weighed and placed on a heating pad. The skin of the right dorsum was shaved and disinfected with povidone-iodine scrub then wiped with gauze soaked in 70% isopropyl alcohol. Animals were positioned prone and sterile surgical drapes were used leaving the surgical site exposed. The inferior angle of the right scapula, the right hip joint, and the spine processes were palpated, then the region of implant insertion was centered between the shoulder and pelvis girdles, 2cm lateral to the midline. Two 1cm incisions were made in the right dorsal skin—1 cm proximal and distal to the designated implant insertion area—running perpendicular to the long axis of the dorsum. After skin incisions proper hemostasis was performed and blunt dissection of the subcutaneous tissue was conducted to create a tunnel between the two incisions. Using a blunt hook, the silk implant was introduced in the cephalic incision and driven into the tunnel by pulling the hook in the caudal direction. After ensuring proper positioning of the silk implant, hemostasis was performed and the incisions were closed using skin clips. Analgesia was provided using sustained-release buprenorphine at a dose of 1.2 mg/kg subcutaneously at the time of anesthesia induction, then repeated every 72 hours as needed.

Specimen Harvesting:
Once euthanized, the animals were dissected to harvest the dorsal skin for histology studies and mechanical testing. The area of skin on the right side was identified using the scars of the surgical procedure and a rectangular area was outlined measuring 5cm in length and 2.5cm in width. A mirror image area on the left side with the same dimensions was also outlined. Both skin areas were harvested using a combination of sharp incision at their outlines and blunt dissection at their bases. Care was taken to dissect the skin base in the plane between the superficial and deep fasciae. After harvesting, specimens for histologic studies were collected from each sample and were fixed immediately. The remainders of each sample were frozen and stored at -4^{o} Celsius for mechanical testing.

Histological studies:
Skin specimens were fixed in 10% neutral buffered formalin for 48 h. The fixed specimens were processed with ethanol and xylene and then embedded in paraffin on an automatic pro-

cessor. Paraffin blocks of each specimen were then sectioned baked on glass slides at 60 °C and then stained with hematoxylin and eosin. Additionally, sections from six of the twelve total samples were stained for alpha-smooth muscle actin.

Images of the H&E stained skin sections were captured at a magnification of 5x using QCapture software and used for quantitative evaluation of skin thickness. All images were imported to ImageJ software and were scaled using an image of the legend micrometer captured at the same magnification. The distance from the superficial aspect of the surface epithelium to the deep aspect of the subcutaneous tissue was measured at three different points and then averaged.

Mechanical testing:

Specimens were thawed to room temperature on testing day. Rectangular skin specimens from the intervention and control sides, further reduced to 2cm in width and 3cm in length, were mounted to a servo-hydraulic testing system (model 8511, Instron, Norwood, MA, USA) using clamps. The functioning length of specimen between clamps was 2.5cm. Specimens were tensioned to 1 N and then loaded at a rate of 1mm/sec until failure.

Results:

Macroscopic evaluation of the surgery site after 10 weeks demonstrated appropriate level of scarring that was consistent across intervention samples with no additional deviations from the control side. Palpation of the skin indicated firm slippery subcutaneous swelling at the silk implant site. All animals appeared healthy with no signs of infection or ulceration.

Histology assessment of all twelve samples with hematoxylin and eosin stain demonstrated silk implants embedded underneath the striated muscle of the deep subcutaneous panniculus carnosus. The staining revealed qualitative differences in foci surrounding the silk implant with the intervention group containing areas of denser connective tissue with higher ratios of nuclei. A layer of especially dense area could be seen surrounding each implant, likely demonstrating the production of a fibrous capsule. Despite this capsule, the silk implants had substantial fragmentation with interwoven subcutaneous stroma. The mean quantitative skin thickness of the intervention samples was 3.89 mm with a standard deviation of 1.19 compared to the mean skin thickness of the controls of 2.63 mm with a standard deviation of . 391; this difference was statistically significant ($p = .0496$). Lastly, alpha-smooth muscle actin staining of six samples yielded high concentrations of stain in the areas surrounding the silk implants which contrasted with sparse staining in the control group.

Mechanical testing provided tensile stiffness (N/mm) and maximum load (N) for a total of 12 samples, 6 intervention samples and 6 control samples. The mean tensile stiffness for the intervention samples was 15.60 N/mm with a standard deviation of 3.25. The mean tensile stiffness for the control samples was 16.86 N/mm with a standard deviation of 7.39. The difference in mean tensile stiffness between these values was not statistically significant ($p = . 515$). The mean maximum load for the intervention samples was 167.34 N with a standard deviation of 39.13. The mean maximum load for the control samples was 177.06 N with a standard deviation of 64.79. The difference in mean maximum load between the intervention and control groups was not statistically significant ($p = .395$).

Discussion:

This study provides further evidence of the biocompatibility of subcutaneous silk implants. There were no grossly observable skin differences between the intervention and control groups beyond typical surgical site scarring. The silk implants did not appear to incite a host immune response that would lead to ulceration or rejection of the material. Histologic assessment of fragmentation within the silk implants suggests a slow biodegradability of the material within the body. While some fracturing may be artifact from the histology slides preparation process, the visibly interwoven stroma indicates a level of inherent decomposition.

The histologic assessment further suggests that silk implants may induce a robust subcutaneous fibrotic reaction. The dense areas visible on the hematoxylin and eosin staining of the intervention group is suggestive of activated fibroblasts surrounded by increased deposition of collagen and other fibers. The high concentration of alpha-smooth muscle actin stain surrounding the implant further supports the presence of activated myofibroblasts.[xv] This finding corroborates descriptions of abundant connective tissue and increased vascularization resulting from silk implants in burn wounds.[xvi] Such a reaction could help promote subcutaneous attachment for percutaneous osseointegrated prostheses and help extend their longevity. The opposite corollary has already been demonstrated for percutaneous devices, where models of poor vascularity lead to greater rates of infection and overall implant failure.[xvii] Other research has also demonstrated that greater subcutaneous attachment to an implant correlates to improved longevity, based upon successful design changes including alterations to surface porosity or the incorporation of an internal flange.[xviii],[xix],[xx]

The purpose of modulating stiffness at the skin implant interface is to bridge the gap between the mechanical properties of the skin with those of the post, minimizing the shearing forces at the skin-implant interface which prevent epithelial cell attachment to the implant. Previous work has demonstrated that maintaining stability at the interface through immobilization provides greater opportunity for tissue attachment with subsequent stabilization of the percutaneous passage.[xxi],[xxii] By increased attachment to the device, the epithelial layer would not continue the well characterized downgrowth that leads to marsupialization with the subsequent opportunity for bacteria to enter the bone.[xxiii] We hypothesized that the silk implants would induce fibrotic change that would lead to increased stiffness; however, mechanical testing demonstrated no statistical difference with respect to either tensile stiffness or tensile load to failure. These mechanical testing results are surprising in the face of histologic evidence of fibrosis combined with the observed increased thickness of the intervention samples. Given the loose organization of the subcutaneous layer and the placement of the implant underneath the deep subcutaneous panniculus carnosus, it is feasible that the observed magnitude of fibrosis in this layer did not directly translate to changes in the mechanical properties of the combined epidermal, dermal, and subdermal layers.

Conclusion:

This work demonstrated the suitability of a subcutaneous silk protein scaffolding in affecting change in the skin of Sprague-Dawley rats. The silk implants were biocompatible and demonstrated minimal degradation over the course of 10 weeks suggesting their suitability for longterm subcutaneous enhancement. Histologic studies provided strong evidence of a robust fibrotic reaction in the areas surrounding the implant which portends a possible mechanism

for increasing subcutaneous attachment to a percutaneous prosthesis, and measurement of the epidermal, dermal, and subcutaneous layers showed skin thickening that could aid the longevity of the corresponding skin implant interface. However, these fibrotic and physical changes did not manifest as recognizable differences in the measured mechanical properties of tensile stiffness and maximum load.

Permutations of experimental design could help elucidate the mechanical property changes in the skin following silk scaffolding emplacement. Shifting the position of the implant superficially may result in a significant change in tensile strength or stiffness, however such a placement will be technically difficult given the minimal plane of surgical dissection. An alteration on the design could include the injection of a liquid form of the silk material that distributes vertically, thereby having a greater impact on mechanical properties across all layers. Such a design would also simplify the procedure of conditioning the skin for future surgeries.

Subsequent research could begin leveraging the results of this study toward application to percutaneous osseointegrated prostheses. The next step would use an animal model to evaluate the silk scaffolding in the setting of a surgically implanted percutaneous titanium rod. Analyzing the rate of infection, mechanical characteristics, and histologic reactions of this model would demonstrate the efficacy of the silk scaffolding while setting a baseline for future modifications involving embedded medications. Incorporation of medications such as antibiotics or growth factors demonstrates the ultimate value of the slowly degrading silk proteins which will use such medications to prevent infections or encourage attachment at the skin implant interface.

Subsequent Project:

Study objective: Use silk scaffolding to achieve a skin-implant interface that enhances the integrity of the integumentary system and protects the body against external pathogens.

Study Design:
Adult male and female Sprague-Dawley rats (aged 13 weeks) will be subjected to surgery to insert transcutaneous implants through 1cm skin defect at the inter-scapular area. Flanged disc shaped titanium implants will be used with different surface characteristics and silk scaffolding according to the following study groups.

Group 1: medical grade polished titanium implants (n=8).
Group 2: medical grade porous surface titanium implants (n=8).
Group 3: medical grade porous surface titanium implants with surrounding silk ring (n=8).

Animals from the three groups will be euthanized six weeks after surgery. The implanted discs and surrounding soft tissue will be harvested for histology (n=2 per group) and mechanical testing (n=6 per group).

Surgical Procedure:
Following anesthesia, animals will be weighed for baseline then placed on a heating pad for further preparation. The skin of the animal dorsum will be shaved and disinfected with povidone-iodine scrub then wiped down with gauze soaked in 70% isopropyl alcohol. Sterile surgical drapes will be used to cover the animal leaving the surgical site exposed. A 1cm diameter biopsy punch will create a skin defect at the inter-scapular area. Additional incisions at the skin edge can be used for the purpose of implant insertion. A pocket will be created by blunt dissection in the subcutaneous space and implants will be inserted with the flanged base completely buried under the skin and its external part protruding from the skin. Skin clips will be used to close any incisions made. Dressing will be applied around the protruding part of the implant, and will be secured using adhesive tape to protect against implant dislodgment.

Outcome Measurements:

Rate of infection:
Animals will be observed for signs of infection including swelling, erythema, and discharge. Should any of these signs happen, a swab for gram stain and culture will be used to identify the causative pathogen. The rate of infection will be calculated and compared between the three groups of the study.

Mechanical testing:
Specimens including the implanted discs and surrounding soft tissue will be subjected to shear load to failure to evaluate the strength of skin-implant interface.

Histological studies:
To avoid disruption of the skin-implant interface, the whole block made of the harvested implant with the surrounding skin specimens will be fixed in 10% neutral buffered formalin then embedded in resin. Coronal sections will be cut using a diamond saw and then ground and polished to the desired thickness. The resulting sections will be stained with hematoxylin and

eosin. Quantitative analysis will be performed to measure epithelial downgrowth and the degree of epidermal, dermal, and subdermal implant attachment.

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