Silk-Based Injectable Biomaterial as an Alternative to Cervical Cerclage: An In Vitro Study

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Abstract

Objective: New therapies to prevent preterm birth are needed. Our objective was to study an injectable biomaterial for human cervical tissue as an alternative to cervical cerclage. **Study Design:** Human cervical tissue specimens were obtained from premenopausal gynecological hysterectomies for benign indications. A 3-part biomaterial was formulated, consisting of silk protein solution blended with a 2-part polyethylene glycol gelation system. The solutions were injected into cervical tissue and the tissue was evaluated for mechanical properties, swelling, cytocompatibility, and histology. **Results:** The stiffness of cervical tissue more than doubled after injection ($P = .02$). Swelling properties of injected tissue were no different than native tissue controls. Cervical fibroblasts remained viable for at least 48 hours when cultured on the biomaterial. **Conclusions:** We report a silk-based, biocompatible, injectable biomaterial that increased the stiffness of cervical tissue compared to uninjected controls. Animal studies are needed to assess this biomaterial in vivo.

Keywords

cerclage, cervix, injectable biomaterial, silk, preterm birth

Introduction

Spontaneous preterm birth is a significant public health problem, complicating more than 12% of the pregnancies in the United States¹ and resulting in \$26 billion in health care costs.² Preterm birth before 32 weeks is the most frequent cause of neonatal mortality³ and is a common cause of long-term neurodevelopmental disability and cerebral palsy.⁴ Although preterm birth is a complex, multifactorial disorder, a dysfunctional cervix is associated with a significant number of preterm deliveries. As cervical shortening is an important risk factor for preterm birth,^{5,6} a short cervix is used to screen patients for therapy to prevent preterm birth.^{7,8} There are currently 2 treatment alternatives when cervical dysfunction is suspected, progesterone supplementation and cervical cerclage.⁹ Because these treatments are not effective for many patients, there is a need for alternative therapies to improve care for women at risk for preterm birth.

In many cases, the pathophysiology of cervical dysfunction is related to weakened biomechanical properties of the cervical stroma.¹⁰ Fibrous connective tissue of the cervical stroma undergoes complex remodeling in preparation for labor.¹¹ Cerclage therapy aims to prevent preterm shortening and insufficiency by providing support to weakened tissue. Although placement of a cerclage is effective in some patients,¹² it has not been shown to reduce the incidence of preterm birth in other cohorts of women.¹³ A cerclage does not

prevent excessive tissue softening, which is the primary pathophysiology of cervical dysfunction. A therapy that enhances the biomechanical properties of the cervix by increasing tissue stiffness could be more effective than cerclage therapy.

Silk fibroin is a naturally derived, fibrous protein that displays remarkable mechanical properties, chemical versatility, and biocompatibility.¹⁴ The mechanical properties of silk fibers arise from the self-assembly of many small crystalline β -sheet structures via intramolecular and intermolecular hydrogen bonding in combination with less organized but hydrogen-bonded domains.^{15,16} Gelation of silk fibroin protein solution can be induced by sonication, physical agitation, alcohol dehydration, and electrical current.¹⁷⁻¹⁹ In addition, the physical properties of silk

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Figure 1. Biomaterial formation, gross morphology, and histology. A (left), The 2-step gelation process is shown. Mixing of the 2 solutions results in a chemically cross-linked gel due to the reaction between the thiol and the maleimide functional groups. Further stabilization arises due to β -sheet formation in silk, which occurs when the silk-polyethylene glycol (PEG) is exposed to methanol or ethanol (white color) but not to water or phosphate-buffered saline (PBS). B (upper right), After injection, the cervical tissue appears larger due to the increase in mass. The silk-polyethylene glycol appears as a translucent, white material on the tissue. C (lower right), Hematoxylin and eosin histology of cervical tissue after injection shows integration of the silk-polyethylene glycol biomaterial (purple) into the tissue. For reference to color, please see online version.

materials can be tuned to meet functional demands by blending with other macromolecules such as tropoelastin (increased elasticity)²⁰ or hyaluronan (biological signaling).²¹ We recently described a 2-part, silk-polyethylene glycol (PEG) biomaterial for use as a tissue sealant.²² This novel biomaterial rapidly gelled after mixing due to the covalent cross-links that formed between the chemically active functional groups on the PEG. In addition, the material was induced to form β sheets from the silk components to enhance the mechanical properties. This silk-PEG biomaterial showed improved mechanical properties and decreased swelling compared to a commercially available PEG sealant.²²

The objective of this study was to investigate the ability of an injectable, silk-PEG biomaterial to increase the stiffness of cervical tissue *in vitro*. The biomaterial was injected into human cervical tissue and studied for mechanical properties, gelation, swelling, and cytocompatibility. This *in*

vitro study is a first step toward a future goal of developing an injectable biomaterial to serve as an alternative to cervical cerclage.

Materials and Methods

Cervical Tissue

Cervical specimens were obtained from premenopausal women after hysterectomy for benign indications ($N = 8$). Informed consent was obtained. Immediately after the removal of the uterus and cervix, a circumferential portion of the cervix between the internal and the external os was removed and placed in ice cold saline (Figure 1B). The specimens were used fresh ($N = 1$) or snap frozen ($N = 7$) in liquid nitrogen and stored at -80°C . Institutional review board approval was obtained from the Tufts Medical Center prior to study initiation.

Figure 2. Swelling properties. Swelling properties of tissue injected with polyethylene glycol (PEG) and silk were no different than the native tissue controls. Swelling was significantly increased ($P < .01$) in the tissue injected with PEG alone.

Silk Fibroin Extraction

Silk fibroin protein was purified as previously described.²³ Briefly, *Bombyx mori* cocoons (Tajima Shoji Co, Ltd, Japan) were cut into 1-cm pieces and boiled in an aqueous 0.2 mol/L sodium carbonate solution for 30 minutes. The fibrous silk was solubilized in 9.3 mol/L lithium bromide at 60°C for 4 hours and dialyzed against distilled water for 72 hours to obtain a 6% (weight/volume [w/v]) silk solution. To increase the concentration above 6%, silk solution was dialyzed against a 10% (w/v) PEG solution as previously described.²⁴ Purified silk fibroin solution was stored at 4°C.

Biomaterial Preparation

Synthesis of the silk-PEG biomaterial followed a previous report.²² Briefly, a 3-part biomaterial was formulated. Purified silk fibroin protein solution was blended with a 2-part PEG gelation system. The chemically active PEG gelation system consisted of 4-arm 10 kDa PEG maleimide (Nanocs, New York, New York) and 4-arm 10 kDa PEG thiol (Creative PEGWorks, Winston-Salem, North Carolina). Solution A was 10% w/v PEG maleimide in the silk solution. Solution B was 10% w/v PEG thiol in the silk solution. The concentration of the silk solution varied from 6% to 15% according to the experimental protocol. A range of silk concentrations was chosen because higher concentrations of silk (ie, 15%) result in stiffer properties compared to lower concentrations (6%), which allows tuning of the final composition to meet the clinical need. Solutions were vortexed at room temperature to promote even mixing. For controls, PEG maleimide and PEG thiol were dissolved in distilled water (10% w/v) with no silk protein.

Gel Formation and Injection Into Tissue

The 2 solutions (1 mL each) were placed in a double-barrel syringe (4B19, RDS/Acuflow, Holliston, Massachusetts) fitted

with a mixing tip (2 × 8 blunt mixer, RDS/Acuflow; Figure 1A). A 20-gauge needle was bonded to the mixing tip. Gelation occurred in 2 steps.²² Initial gelation occurred within seconds after mixing as covalent cross-links formed between the maleimide and thiol functional groups. A physical cross-linking step occurs subsequently over a longer period of time and involves β sheet formation in the silk protein chains (Figure 1A). To accelerate β sheet formation, tissue-silk-PEG was placed in absolute ethanol for 10 minutes²⁵ and then stored in phosphate-buffered saline (PBS) for experiments.

Gross Morphology and Histology

The silk-PEG biomaterial (silk protein 7%) was injected into cervical tissue. Gross images of the tissue before and after injection were obtained. The tissue was then formalin fixed and paraffin embedded. To obtain a subjective assessment of how the silk-PEG biomaterial integrates into the tissue after injection, hematoxylin and eosin (H&E) staining was performed using standard protocols.

Swelling Properties

Swelling properties were studied because PEG-based biomaterials are known to swell in vivo.²⁶ Cervical tissue was cut into cylinders (8 mm diameter) using a punch biopsy (33-37, Miltex; Figure 2). Solutions of PEG-thiol and PEG-maleimide were made with 10% and 15% silk as described above. Cervical tissue specimens were injected with PEG only, PEG in 10% (w/v) silk, and PEG in 15% (w/v) silk. To determine the amount of silk-PEG biomaterial that was injected, the specimens were weighed before and after the injection. The specimens were placed in alcohol for 10 minutes followed by 1 × PBS, pH 7.4 for 15 minutes. Excess fluid was blotted and the initial weight was recorded. The tissue-silk-PEG samples were stored in 12-well plates, covered with 1 × PBS, pH 7.4, incubated at 37°C. The samples were weighed at 10 minutes, 20 minutes, 40 minutes, 1 hour, 2 hours, 8 hours, 24 hours, and

48 hours. Swelling was calculated at each time according to the following formula: The percentage increase in sample weight, W_{increase} , was calculated as:

$$W_{\text{increase}} = 100(1 + (w_t - w_o)/w_o), \quad (1)$$

where w_o is the initial weight and w_t is the weight measured after a given amount of time.

Mechanical Testing

Cervical tissue specimens were thawed in PBS at room temperature. The tissue was sliced with a blade so that the top and bottom surfaces were parallel. Mechanical testing was performed in a custom designed acrylic fixture filled with PBS (Figure 3A). Testing was performed by tissue indentation with a solid rod. A universal material testing machine (Zwick Z2.5/TS1 S, Ulm, Germany) with a 20 N load cell was used to collect the indentation data. The machine controlled the displacement of the tissue according to user-defined testing protocols. Optimal loading protocols were determined in preliminary experiments. Each cervical specimen was preloaded at the start of the test with a small indentation (4–8 mN). The specimen was then subjected to 20% indentation at 0.1 mm/s and a load–displacement curve was recorded. This cyclic loading protocol was repeated 3 times at each test site to assess repeatability. The test site was marked and the tissue was injected with 0.5 to 1.0 mL of silk–PEG biomaterial (silk 7% w/v). The treated specimens were placed in alcohol for 10 minutes followed by 1× PBS for 15 minutes. Repeat indentation testing was then performed at the same site. Peak force and change in the thickness of the tissue was measured.

Cytocompatibility Assay

Human cervical fibroblasts were cultured from fresh cervical tissue using an explant technique as previously described.²⁷ Briefly, minced cervical tissue was cultured in Dulbecco Modified Eagle Medium, 10% fetal bovine serum, and antibiotics. After 10 days, cervical fibroblasts grew out from the explants. The fibroblasts were culture expanded and cryopreserved using standard techniques.

The fibroblasts were cultured on 2 surfaces: silk–PEG only (control) and cervical tissue injected with silk–PEG. For cervical tissue, a cervical biopsy (3 cm × 3 cm × 0.3 cm) was harvested from the operating room and used fresh. Silk–PEG (silk protein 7% w/v) was injected into cervical tissue. The tissue–silk–PEG was divided along the long axis to expose a surface with tissue and silk–PEG. The exposed surface was seeded with 100 000 fibroblasts and placed in culture media. As a control, the silk–PEG biomaterial (no tissue) was seeded with fibroblasts. The seeded tissue and control were incubated at 37°C for 48 hours. Cell viability was assessed using the Live/Dead viability/cytotoxicity kit (Molecular Probes, Life Technologies, New York) by incubation with 2 μmol/L ethidium and 1 μmol/L acetomethoxy derivative of calcein (calcein AM) at room temperature for 30 minutes. Uptake of

calcein AM occurs in live cells and is subsequently converted to calcein, giving the cells a green fluorescence. Ethidium enters damaged cells and produces a red fluorescence by binding to nucleic acid. Cell morphology was visualized using inverted fluorescence microscopy (Axiovert S100, Leica Microsystems Inc, Buffalo Grove, Illinois).

Statistical Analysis

Student *t* test was used to analyze continuous variables and a paired *t* test was used to analyze paired samples. Analysis of tissue swelling was assessed using 2-way analysis of variance (GraphPad Prism ver. 5.04, GraphPad Software, San Diego, California). A *P* value <.05 was considered statistically significant.

Results

Cervical Tissue Specimens

A total of 8 patients were consented for removal of cervical tissue specimens posthysterectomy (Caucasian: N = 4, African American: N = 3, Hispanic: N = 1). The age of patients ranged between 37 and 54 years (median = 47 years). All the patients were multiparous and 7 of the 8 patients had a history of at least 1 vaginal delivery. The indications for hysterectomy were fibroid uterus/menorrhagia (N = 6), dysfunctional uterine bleeding (N = 1), and prophylactic due to a history of Lynch syndrome (N = 1).

Gel Formation and Tissue Injection

Rapid gelation (in seconds) of a translucent, white material was observed after solution A and solution B were injected through the mixing tip and 20-gauge needle. Gelation was confirmed by the vial inversion test as previously described (Figure 1A).²² After 10 seconds of injection, gelation of the biomaterial within the mixing tip prevented further injection and a new mixing tip was necessary. Figure 1B shows changes in the shape and size of cervical tissue after injection. Observation of specimens using H&E staining showed that the silk–PEG biomaterial was incorporated into the cervical tissue specimen after injection (Figure 1C).

Swelling Properties

The mean (standard deviation [SD]) increase in tissue weight after injection of the silk–PEG biomaterial was 17% (6.7%). The swelling properties of tissue injected with silk–PEG biomaterials (10% and 15% w/v silk) were not significantly different compared to the native tissue controls (Figure 2). However, tissue swelling was significantly increased when injected with PEG only (*P* < .01).

Mechanical Testing

Indentation testing was performed on cervical tissue from 4 participants before and after the injection of silk–PEG biomaterial (7% w/v silk) at predetermined sites on the tissue

Figure 3. Mechanical testing. A (upper left), Setup for indentation testing with the cervical tissue specimen placed in phosphate-buffered saline (PBS). Indentation was performed in the same spot before and after tissue injection. B (upper right), A sample load–unload cycle on a cervical tissue specimen shows a consistent response between the testing cycles ($n = 3$ cycles). Of note, the sample was preloaded by 4 to 8 mN prior to indentation. C (lower left), The peak force needed to compress the cervical tissue by 20% increased significantly after injection of the silk–polyethylene glycol (PEG) biomaterial. D (lower right), A positive dose response was seen. As more silk–PEG biomaterial was injected, the peak force ratio significantly increased ($P = .002$).

(Figure 3A). The load–unload response was consistent between the testing cycles (Figure 3B) indicating the loading protocol did not damage the tissue. The maximum force needed to indent the tissue by 20% more than doubled after the injection of cervical tissue with silk–PEG biomaterial (Figure 3C). The mean (SD) ratio of maximum force to injected/uninjected tissue was 2.5 (1.4; $P = .02$).

To assess the dose–response effect, the maximum force was plotted as a function of injected biomaterial (Figure 3D). The amount of injected biomaterial was quantified by measuring the thickness of the tissue before and after injection and calculating the percentage thickness increase (0% corresponds to no change in thickness; 100% corresponds to doubled thickness). Figure 3D shows a dose–response effect. As more biomaterial was injected, the peak force significantly increased and varied linearly with the increase in thickness ($R^2 = .87$, $P = .002$).

Cytocompatibility Assay

After 48 hours, few nonviable fibroblasts (red fluorescence) were seen on the tissue–silk–PEG and silk–PEG biomaterials. Viable fibroblasts (green fluorescence) were visualized on both tissue–silk–PEG and silk–PEG biomaterials (Figure 4). However, the morphology of the viable fibroblasts appeared different. Fibroblasts on silk–PEG biomaterial appeared rounded, probably because there were fewer cell attachment sites.²² In contrast, cells on tissue–silk–PEG displayed both rounded and spindle-shaped morphology.

Discussion

In this study, we synthesized an injectable silk–PEG biomaterial that increased the stiffness of human cervical tissue

Figure 4. Cytocompatibility. Cervical fibroblasts are viable (green cells) when cultured for 48 hours on cervical tissue injected with silk–polyethylene glycol (PEG) biomaterial. Viable cervical fibroblasts display a rounded morphology on the silk–PEG biomaterial and spindle-shaped morphology on cervical tissue (inset). On the silk–PEG biomaterial alone (control), the cervical fibroblasts have a rounded morphology (far right panel). For reference to color, please see online version.

when tested *in vitro*. The silk–PEG biomaterial gelled rapidly within the tissue and was not cytotoxic to primary cervical fibroblasts. Swelling of the tissue–silk–PEG was not different from the cervical tissue controls. These results are a promising first step for the development of an injectable biomaterial that could serve as an alternative to cervical cerclage.

An effective alternative to cerclage therapy could have an important impact on the clinical practice. Cervical cerclage is indicated in 3 clinical settings: (1) a known history of cervical insufficiency, (2) a prior history of preterm birth with a short cervical length,¹² and (3) a dilated cervix in the mid-trimester.^{28,29} Although placement of a cerclage is more effective than expectant management in these settings, miscarriage or preterm birth can still occur even though cerclage is present. Lack of efficacy is seen in the following settings. First, cerclage displacement or cervical laceration³⁰ can occur which leads to reduced efficacy. Second, cerclage is ineffective in the setting of multiple gestations.³¹ Third, intrauterine infection or utero-placental bleeding can occur even though a cerclage is present. Finally, cervical dilation often begins near the internal os of the cervix, but cerclage placement at this location can be technically challenging from a vaginal approach. We speculate that an injectable therapy could address one or more of these limitations of cerclage, which would be an exciting clinical development.

The rationale for developing an injectable biomaterial was to improve the properties of the cervical tissue so that it could provide better support than a cerclage. The cervical extracellular matrix (ECM) is composed of fibrous connective tissue. The most important constituent of the ECM is fibrillar collagen.¹¹ Key ECM molecules that affect the collagen network include hyaluronan,³² proteoglycans,³³ and water.³⁴ In prior work, we have shown the macroscopic mechanical properties of cervical tissue arise from this fibrous ECM.^{11,35} In this study, we demonstrated a prototype biomaterial that integrated into the cervical tissue and increased its stiffness. In contrast, a cervical cerclage does not change the properties of cervical tissue. Hence, a biomaterial that stiffens the tissue could provide better

support to the weakened tissue compared to a cerclage. In addition, an injectable biomaterial could be easier to place than a cerclage because a straight needle has improved access to the upper part of the cervix compared to a curved needle used for cervical cerclage placement. We anticipate this biomaterial will be injected circumferentially near the level of the internal os.

Silk and PEG were chosen for the prototype because both materials are biodegradable, biocompatible, and approved by the Food and Drug Administration for use in the body. Silk sutures are classified as nonabsorbable because they retain tensile strength at least 60 days *in vivo*.³⁶ The rate of degradation of silk biomaterials can be accelerated or delayed by changing the method of purification, silk morphology, and silk concentration.³⁷ In addition, the biocompatibility of silk films in rats were found to be comparable to other commonly used biomaterials.³⁸ CoSeal is a PEG-based surgical sealant approved to achieve hemostasis in the setting of vascular reconstructions. The rationale for combining silk with PEG was to improve both the mechanical properties and decrease the swelling of PEG alone.²²

An injectable biomaterial for cervical support needs to have a well-defined degradation profile. In the mid-trimester and early third trimester, the biomaterial should stiffen the tissue to prevent cervical shortening. Close to term, the biomaterial should be degraded to allow normal cervical dilation. We have previously shown that complete degradation of silk scaffolds in rats varied from 2 months to 1 year depending on the purification process.³⁷ The silk protein concentration also affected the degradation profile.³⁷ However, previous degradation studies have not been performed during pregnancy. An important future design goal will be to characterize time-dependent degradation *in vivo* with the aim to meet the requirements of pregnancy.

In several clinical settings, such as intrauterine infection or unstoppable preterm labor, it is desirable to promote delivery. An injectable biomaterial needs to allow cervical dilation in these settings and provide support when cervical shortening is not desired. Judicious choice of biomaterial composition and

processing will be important to achieve these goals. If excessive tissue stiffening is encountered in preclinical models, it will be important to modify the biomaterial to achieve a better safety profile.

Our study is novel and provides the basis for future work in developing an alternative therapy for cervical insufficiency. The silk-PEG biomaterial stiffened nonpregnant human cervical tissue, which likely represents a worst-case scenario since nonpregnant cervical tissue is significantly stiffer than cervical tissue from pregnancy.³⁴ In addition, the biomaterial was not cytotoxic to primary human cervical fibroblasts, which are the most plentiful cells in the cervical stroma. We did observe a change in the typical morphology of fibroblasts. Fibroblasts typically have a spindle appearance but the cells appeared rounded when cultured on the biomaterial. The significance of the change in morphology is unclear and will be the subject of future in vivo studies.

An important limitation of our study was the need to accelerate β sheet formation in the silk-PEG with exogenous ethanol. This step was necessary because preliminary experiments showed silk-PEG had inadequate mechanical properties in the absence of β sheet formation. This limitation could be addressed by alternate methods of inducing β sheet formation in silk including sonication, physical agitation, and electrogelation.¹⁷⁻¹⁹ The design goal will be an injectable formulation that safely gels after injection. This study was also limited because in vivo data were not presented. However, our goal was to develop an initial prototype that could be used for future in vivo experimentation. Finally, development work is necessary to optimize biomaterial synthesis, mechanical properties, and storage prior to in vivo experimentation.

In summary, we report a biocompatible, silk-PEG biomaterial that stiffened the cervical tissue in vitro. This study is a first step toward a long-term goal of developing an injectable biomaterial as an alternative to cervical cerclage for treatment of a short cervix or cervical insufficiency. Further studies are needed to test this biomaterial in vivo.

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Authors' Note

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Declaration of Conflicting Interests

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