Biocompatibility of a Sonicated Silk Gel for Cervical Injection During Pregnancy: In Vivo and In Vitro Study

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Abstract

Objective: To evaluate the biocompatibility of silk gel for cervical injection. **Study Design:** Silk gel was injected into the cervix of pregnant rats on day 13 ($n = 11$) and harvested at day 17. Histology of silk gel was compared with suture controls. Also, human cervical fibroblasts were cultured on silk gel and tissue culture plastic (TCP) in vitro. Cell viability, proliferation, metabolic activity, gene expression (*COL1A1*, *COL3A1*, and *COX2*), and release of proinflammatory mediators (interleukin [IL] 6 and IL-8) were evaluated. **Results:** In vivo, a mild foreign body response was seen surrounding the silk gel and suture controls. In vitro, cervical fibroblasts were viable, metabolically active, and proliferating at 72 hours. Release of IL-6 and IL-8 was similar on silk gel and TCP. Collagen and *COX2* gene expression was similar or slightly decreased compared with TCP. **Conclusions:** Silk gel was well tolerated in vivo and in vitro, which supports continuing efforts to develop silk gels as an alternative to cervical cerclage.

Keywords

cerclage, cervix, injectable biomaterial, silk, preterm birth

Introduction

Preterm birth prior to 37 weeks of gestation affects more than 12% of pregnancies in the United States¹ and has broad impact on both a societal and an individual level. Health care costs related to preterm birth exceed US\$26 billion.² Globally, preterm birth is the leading cause of child mortality in high-income countries.³ Those preterm infants that do survive are at significant risk of multiple long-term health complications including developmental disability, psychiatric sequelae, visual and hearing impairment, chronic lung disease, and cardiovascular morbidity.⁴⁻¹¹ In addition, the families of these children face significant financial and emotional strain.¹² Unfortunately, the efficacy of existing therapies for preterm birth prevention is modest at best; a small decrease in the incidence of preterm birth can be expected with optimal usage of existing therapies.¹³

Currently available therapies to prevent preterm birth have important limitations. Although progesterone supplementation is an important therapy for women with a history of spontaneous preterm birth, it prevents recurrent preterm birth in only one-third of the cases.¹⁴ Although vaginal progesterone is used for women with cervical shortening,^{15,16} the Food and Drug Administration (FDA) rejected a new drug application for this indication citing lack of efficacy among women in the United States.¹⁷ Cervical cerclage is effective in women with cervical

shortening and a history of preterm birth but cerclage is not effective if there is no history of preterm birth.^{18,19} In addition, cerclage can cause complications such as bleeding, rupture of membranes, and cervical trauma.²⁰ In populations tested to date, neither progesterone nor cerclage is effective in women with multiple gestations.^{21,22} These limitations have prompted the call for new therapies to prevent preterm birth.²³

Cervical dysfunction is a prominent feature of preterm birth in many women²⁴ and several existing therapies aim to prevent cervical dysfunction. Both cerclage and pessary²⁵ act to provide improved support for cervical tissue with the goal of preventing undesired shortening and dilation. However, these therapies do

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not address excessive tissue softening, which may be central to the pathogenesis of the disease. A therapy that prevents cervical softening could be more efficacious than either cerclage or pessary. We previously presented an injectable, silk-based biomaterial for stiffening of cervical tissue.²⁶ This biomaterial was composed of FDA-approved materials (silk and polyethylene glycol [PEG]) and stiffened human cervical tissue *in vitro*. However, the gelation mechanism required exogenous ethanol and no data on biocompatibility were presented.

The purpose of the present study was to evaluate the biocompatibility of an injectable silk gel that did not require exogenous ethanol for gelation. For this purpose, the silk gel was sonicated with high-intensity ultrasound prior to injection, which accelerated spontaneous gelation.²⁷ We hypothesized that the silk gel would show comparable biocompatibility to suture materials used for cervical surgery. We further hypothesized that the silk gel would be cytocompatible with cervical fibroblasts and immune responses would be similar to cell responses on tissue culture plastic (TCP) controls.

Materials and Methods

Silk Fibroin Extraction

Silk fibroin protein was purified as described previously.²⁸ 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 dissolved 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% (w/w) purified silk solution. The silk solution was stored at 4°C until use.

Silk Gel Preparation

The 6% silk solution was diluted to 3% (w/w) and filter-sterilized (#565-0020, Thermo Scientific Nalgene, Waltham, MA). The sterile silk solution was placed in a sterile dialysis cassette (10 000 MWCO, #66456 Slide A Lyzer, Pierce Biotechnology, Rockford, Illinois) and concentrated by slow evaporation of water in a cell culture hood for up to 24 hours, yielding a concentrated sterile silk solution of 6%-10% (w/w). Although a pure silk solution will gel spontaneously, it was necessary to accelerate this transition using high-intensity ultrasound.²⁷ Using a 3-mL syringe, 1.5 mL of silk solution was sonicated with Branson 450 Sonifier (Branson Ultrasonics, Danbury, CT) and a 1/8-in diameter tapered microtip. The microtip was sterilized with ethanol solution and sonication was performed in a cell culture hood (Figure 1). Solutions were sonicated for 10 to 15 seconds at 15% amplitude at 20 kHz frequency while surrounded by an ice-cold bath. Sonication times were chosen to achieve gelation within 24 hours of sonication. A range of sonication times were needed (10-15 seconds) because of known heterogeneity in the silk solution.

In Vivo Biocompatibility

To study short-term biocompatibility of the silk gel, a pregnant rat model was used. Sterile silk solution (9% w/w) was sonicated

for 10 to 15 seconds and stored on ice until injection. Injection was performed 2 to 3 hours after sonication. Preliminary experiments showed all solutions gelled at 12 to 24 hours at 37°C with this sonication protocol. In preliminary experiments, we attempted to perform cervical injections via a vaginal route with the aid of an endoscope but this was not feasible due to poor visualization. Thus, the vaginal route was not used. An abdominal route was used in subsequent animals. General anesthesia was induced and a midline laparotomy was performed on timed pregnant Sprague-Dawley rats ($n = 11$; Charles River, Wilmington, Massachusetts) on gestational day 13. The abdomen was explored and the cervix was brought to the skin surface. Of the 11 rats, 8 rats were injected with silk gel and 3 were used as surgical controls. For the experimental group, approximately 500 μ L of gel was injected under direct visualization into 2 to 3 locations using a 5/8-in 25-gauge needle. To compare the biological response of the silk gel to suture materials used for cervical surgery, a polyethylene terephthalate suture ($n = 1$; 4-0 Mersilene, Ethicon, Somerville, NJ) and a polyglactin 910 suture ($n = 1$; 4-0 Vicryl, Ethicon) was sutured in the cervical stroma (Figure 1). As a negative control, a saline injection ($n = 1$) was performed in a manner similar to the silk gel injections. Animals were sacrificed on gestational day 17. Cervices were harvested via laparotomy, fixed in formalin, and paraffin embedded. Histological evaluation was performed by a veterinary pathologist (LR). The study protocol was approved by the Tufts Medical Center Institutional Animal Care and Use Committee.

Cervical Cell Culture

Cervical biopsies were obtained from premenopausal women after hysterectomy for benign indications as described previously.²⁹ Informed consent was obtained, and institutional review board approval was obtained prior to study initiation. Cells were isolated using an explant culture method as described previously.²⁹ These cells have been shown to have a fibroblast phenotype.³⁰ Cells from passage 4 to 5 were used for all experiments. Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic was used as a standard medium.

In Vitro Cytocompatibility

Cervical fibroblasts were cultured on silk gels and cell responses were compared to cell responses on TCP.

Cellular viability. Viability was assessed with a 2-color fluorescence assay (LIVE/DEAD viability/cytotoxicity kit, Life Technologies, Carlsbad, CA). Sterile silk (5.9% and 9.3%) was sonicated for 10 seconds and 150 μ L/well was plated in a 6.5 mm diameter transwell (#3470 Transwell, Corning Inc, Corning, NY). Transwell plates were used for cell culture so that the gels could be removed for microscopy by cutting the Transwell membrane. The silk gelled after 24 hours at 37°C. Cervical fibroblasts were applied to the gel surface at 20 000

Figure 1. In vivo study. Timeline for cervical injections and sonication setup (top, left). In suture controls, squamous metaplasia was seen around Vicryl and Mersilene sutures (top, right). In silk gel, a mild foreign body response was seen (bottom).

cells/cm² (5000 cells per well) and cultured in standard media for 72 hours. After 72 hours, the cells were incubated with calcein AM (1 μ mol/L) and ethidium (2 μ mol/L) for 30 minutes at room temperature and visualized using an inverted fluorescence microscope (Axiovert S100, Leica Microsystems Inc, Buffalo Grove, Illinois). Cell viability and morphology were compared to fibroblasts cultured on silk-PEG gels²⁶ and TCP.

Cellular metabolic activity. Metabolic activity was assessed with the alamarBlue reagent (Invitrogen). Sterile silk was sonicated for 10 seconds and the gel (5.9% and 9.3%) was plated in 24-well plates. After silk gelation, cervical fibroblasts were applied to the surface of the gel (20 000 cells/cm²). Metabolic activity was measured after 5 and 10 days of culture. The 10 $\times$ alamarBlue reagent was diluted with growth medium and 750 μ L was applied to each well following the kit instructions. After 4 hours of incubation, fluorescence was read at 560 nm excitation/590 nm emission and compared to cell culture on TCP.

Cell proliferation. Although cell proliferation could be visualized on the silk and TCP surfaces using the viability assay, it was

desired to quantify this proliferation using a DNA assay. Sterile silk (6.0% and 7.2%) was sonicated for 15 seconds and 200 μ L/well was plated on 6.5-mm Transwell plates (described previously). After gelation, 5000 cells/cm² were applied to the gels. After 24 and 72 hours, the gels were removed from the plates, flash frozen in liquid nitrogen, and stored at -20°C. DNA concentration was quantified using a fluorescent nucleic acid stain (Quant-iT PicoGreen dsDNA kit, Invitrogen). Briefly, gels were minced with scissors in 1 mL digestion buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) with proteinase K 0.5 mg/mL (#19131, Qiagen, Valencia, CA) at 50°C overnight. The digested solution was clarified at 8000 g for 10 minutes and the supernatant was evaluated for DNA concentration. As a control, 5000 cells/cm² were plated on TCP (96-well plates). After 24 and 72 hours, cells were lysed with 0.85 mmol/L Triton X-100 in Tris-EDTA buffer and evaluated for DNA concentration. λ DNA was used as the standard curve.

Proinflammatory mediators. Sterile sonicated silk (7.2% and 9.8%) was plated on 24-well plates. The wells were lined with

sterile aluminum foil to aid removal of the silk gels. After gelation, 5000 cells/cm² were applied to the wells and incubated for 120 hours. Culture media were collected and stored at 4°C. To measure the release of proinflammatory mediators, enzyme-linked immunosorbent assay was performed with human interleukin (IL) 6 (D6050, Quantikine ELISA, R&D Systems, Minneapolis, Minnesota), human IL-8 (D8000C, Quantikine), and prostoglandin E2 (PGE2; KGE004B, Quantikine) following the manufacturer's protocol. Proinflammatory mediator expression was normalized against DNA concentration. Briefly, the gels were removed from the wells, flash frozen on dry ice, and the aluminum foil was carefully peeled away. A 6-mm punch hole biopsy was used to obtain a cutout from the center of the gel, which was digested and evaluated for DNA content as described previously. Release of proinflammatory mediators on silk gels was compared to TCP controls on 24-well plates.

Gene expression. Gene expression of collagen-related transcripts (*COL1A1* and *COL3A1*) and *COX2* (inflammatory response) was compared to gene expression in cells cultured on TCP using quantitative real-time reverse transcription polymerase chain reaction. Briefly, sterile silk (6.0%, 7.2%, and 9.8%) was sonicated for 10 seconds and 150 µL/well was plated on 6.5-mm Transwell plates as described previously. After gelation, cervical cells were applied to the gel surface and cultured for 5 days. Silk gels were removed from the transwell plates, flash frozen in liquid nitrogen, and stored at -80°C until time of assay. Cells cultured on TCP were flash frozen while adherent to the culture plate. Isolation of RNA was performed with the RNeasy Mini kit (#74104, Qiagen).³⁰ Briefly, silk gels were unfrozen in a guanidine-based lysis solution, minced with scissors, and centrifuged. Cells on TCP were lysed directly with the guanidine-based lysis solution and centrifuged. RNA from the supernatant was purified on the RNeasy spin column. Purified RNA was eluted with 50 mL of water and stored at -80°C. The A260–A280 ratio was greater than 2.0 for all samples (Nanodrop 2000, Thermo Scientific). The RNA concentration ranged from 1.0 to 7.7 ng/µL. Reverse transcription reactions and gene expression quantification were performed as described previously with the Stratagene Mx 3000P QPCR System (Stratagene, La Jolla, CA) and TaqMan gene expression assays (Applied Biosystems, Carlsbad, CA).³⁰ The following transcripts were quantified: collagen type 1 (collagen type I, $\alpha 1$ [*COL1A1*], assay ID Hs00164004_m1), collagen type 3 (collagen type III, $\alpha 1$ [*COL3A1*], assay ID Hs00164103_m1), and *COX2* (prostaglandin-endoperoxide synthase 2, assay ID Hs00153133_m1). Gene expression was normalized by the geometric mean of 2 housekeeping genes: β -actin (assay ID Hs99999903_m1) and glyceraldehydes-3-phosphate dehydrogenase (assay ID Hs99999905_m1). Fold change data were normalized to TCP controls. Controls performed without reverse transcriptase revealed no amplification.

Statistical Analysis

Data are expressed as mean values $\pm$ standard deviation. Mean values were calculated from 4 to 6 replicates as indicated.

Figure 2. Viability and proliferation on silk gel surface. Cervical cells were viable on silk-PEG, silk gel, and TCP (green fluorescence). Cell morphology on silk gels appeared similar to TCP controls, which was in contrast to rounded cells seen on silk-PEG (left). PEG indicates polyethylene glycol; TCP, tissue culture plastic.

Comparisons between means were performed using Students *t* test and analysis of variance with Bonferroni multiple comparison test where appropriate (GraphPad Prism ver. 5.04, San Diego, California). Results were considered significant at $P < .05$.

Results

In Vivo Biocompatibility

All animals tolerated the procedure well. None of the animals delivered preterm. Of the 8 animals injected with silk gel, silk was seen in the cervical tissue in 4 (50%) animals and no silk was seen in the other 4 animals. On histology, a mild to moderate granulomatous to eosinophilic inflammatory response was seen surrounding the silk material, consistent with a mild foreign body response (Figure 1). No neutrophil response was seen. A similar foreign body response (mild granulomatous to eosinophilic inflammation) was also seen in the suture controls (Figure 1). In addition, areas of squamous metaplasia were noted in the suture controls, which were not seen in the cervixes injected with silk gel. The saline control was notable for a normal cervical stroma but a mild foreign body response on the uterine serosa, which was consistent with surgical manipulation.

In Vitro Biocompatibility

Cellular viability. At the end of the 72-hour culture period, few nonviable cells were seen (red fluorescence). Viable green cells were seen on all 3 surfaces (Figure 2). However, the morphology of the cells appeared different on different surfaces. Cells cultured on silk-PEG appeared rounded as described previously.²⁶ However, cells cultured on silk gel appeared spindle-shaped, similar to cells cultured on TCP.

Figure 3. Cell responses on silk gel: Metabolic activity: compared with TCP, cellular metabolic activity was significantly decreased on 5.9% and 9.3% silk gels at 5 days of culture ($P < .01$). At 10 days of culture, no difference in metabolic activity was seen between TCP and 9.3% gels but remained significantly decreased on 5.9% gels. Proliferation: cell proliferation (right) was observed on all silk gel surfaces ($P < .01$). No difference was seen between proliferation on silk gels and TCP. Inflammatory mediators: there was no difference in release of IL-6 ($P = .09$) and IL-8 ($P = .3$) from cells on silk gels compared with TCP. Data presented as mean $\pm$ standard deviation of 4 replicates. IL indicates interleukin; TCP, tissue culture plastic.

Cellular proliferation. Measurement of DNA concentration at 2 time points confirmed cellular proliferation on silk gel surfaces (Figure 3). The amount of DNA per well significantly increased from 24 to 72 hours when cervical cells were cultured on 6.0% and 7.2% silk gels ($P < .01$). The DNA concentration was not significantly different between silk gels and TCP.

Cellular metabolic activity. Decreased metabolic activity was seen when cells were cultured on silk gels compared with TCP (Figure 3). At 5 days of culture, the metabolic activity of cells cultured on 5.9% and 9.3% silk gels was significantly less than the metabolic activity of cells cultured on TCP ($P < .0001$). At 10 days of culture, the metabolic activity was significantly less ($P < .0001$) when cells were cultured on 5.9% silk gel versus TCP. At 10 days of culture, there was no difference in metabolic activity when cells were cultured on 9.3% silk gel versus TCP.

Proinflammatory mediators. No significant differences were seen in the release of proinflammatory mediators IL-6 ($P = .09$) and

IL-8 ($P = 0.3$) by cells cultured on silk gels compared with TCP (Figure 3). The release of PGE2 was not detected when cells were cultured on silk gels or TCP.

Gene expression. No differences were seen in expression of *COL1A1* when cells were cultured on silk gels versus TCP (Figure 4). Significantly decreased expression of *COL3A1* was seen on 6% ($P < .01$) and 9% silk gels ($P < .01$) but not 7% gels. Significantly decreased expression of *COX2* was seen on 6% gels ($P < .05$) but no change in expression was seen for 7% or 9% gels when compared to TCP (Figure 4).

Discussion

An efficacious alternative to cerclage therapy could have a major impact on clinical obstetrics. Previous work showed the feasibility of an injectable, silk-based biomaterial to stiffen cervical tissue in vitro.²⁶ Here, we studied the biocompatibility of silk gels in vivo and in vitro. Cervical injections were well tolerated by pregnant rats. Short-term exposure to the silk gel

Figure 4. Gene expression. Expression of *COL1A1* was not different on silk gels or TCP. Expression of *COL3A1* was significantly decreased on 6% and 9% gels but not 7% gels ($P < .01$). Expression of *COX2* was decreased on 6% gels ($P < .05$) but not 7% or 9% gels. Data presented as mean $\pm$ standard deviation of 5 replicates. TCP indicates tissue culture plastic.

provoked a mild foreign body response in the cervical stroma. This response was also seen with suture materials used for cervical surgery. In vitro, cervical cells were viable, metabolically active, and proliferating when cultured on the silk gel surface. Gene expression of collagen transcripts and *COX2* was the same or slightly decreased when compared with TCP. Release of proinflammatory mediators was not increased on silk gels. These results are promising for further development of a silk-based biomaterial as a cerclage alternative.

Although cervical cerclage is an important treatment alternative for many women,^{19,31} there is no accepted animal model for studying cerclage treatment. We chose a rodent model because rodents have been used extensively to study cervical softening during pregnancy.³²⁻³⁴ The rat was chosen because the rat cervix is larger than the mouse cervix. We found cervical injections were well tolerated in the rat and the short-term foreign body response to silk gel was similar to the response seen with cervical sutures. We also observed areas of squamous metaplasia in the cervixes of the Mersilene and Vicryl suture control animals, which was not seen with silk gel. It is known that Vicryl promotes a mild inflammatory response in the rat abdomen³⁵ and squamous metaplasia is a response to chronic inflammation.^{36,37} We speculate that areas of squamous metaplasia may represent increased inflammatory responses to cervical sutures compared with the silk gel. Further studies are needed to confirm this observation.

The silk gel surface was well tolerated by cultured human cervical fibroblasts. No cytotoxicity was seen. We noted some heterogeneity in expression of collagen transcripts (ie, decreased expression of *COL3A1* on 6% and 9% gels but not on 7% gels), which we interpret as due to biologic variability. Overall, expression of collagen-related transcripts was the same or only slightly decreased than that of TCP controls. To study the inflammatory response, we chose to measure IL-6, IL-8, and PGE2, which are cytokines that have been implicated in cervical ripening.³⁸ Release of proinflammatory mediators and expression of the *COX2* gene were the same or decreased on the silk gel surface. Prior studies of human mesenchymal

stem cells³⁹ and human dermal microvascular endothelial cells⁴⁰ cultured on silk surfaces showed similar results—cytotoxicity was not seen, and immune responses were the same or decreased than those of TCP controls. Overall, these results suggest that an injectable silk gel will not be associated with abnormal cellular response in vivo. Also, silk mesh and silk sutures are FDA approved for human use, which is further evidence for its safety. It is important to emphasize the limitations of the rodent model for evaluation of gel safety. Because the rat gestation is 22 days, there was a short period (4 days) between injection and assessment of biocompatibility. In addition, there were important aspects of safety (ie, potential of embolization and potential for excessive tissue stiffening) that were not assessed in the current report. We regard the present report as a positive step toward development of a safe alternative for preterm birth prevention.

A key future goal is to show an improvement in cervical function after injection with a silk gel during an animal model of pregnancy. Improved functional performance will be defined as an increase in tissue stiffness but without an adverse effect on labor. It is necessary to determine the optimal properties of the silk gel to achieve this goal. The properties of silk gels arise from the self-assembly of small crystalline β -sheet structures via intramolecular and intermolecular hydrogen bonding.^{41,42} These properties can be controlled to optimize gel composition and processing to achieve desired functional goals.²⁷ The present study improves on an earlier study²⁶ because no exogenous alcohol was used to accelerate gelation. Of note, future studies to demonstrate an improvement in functional performance will require a larger animal model than the rodent because, in 50% of the animals, no silk gel was seen in the cervical stroma. Larger animals will also allow long-term studies of biocompatibility and biodegradability.

Although ascending infection and inflammation are known to occur with a short cervix, the causal relationship between cervical shortening and infection/inflammation is not well defined. The central hypothesis of the current project is that, in a significant number of patients, cervical shortening is the

first step in the natural history of ascending infection/inflammation. Thus, a therapy that prevents cervical shortening (ie, cerclage, pessary, and injectable gel) could delay spontaneous preterm birth by maintaining an effective barrier against infection. We recognize that an alternate pathogenesis is possible. Ascending infection/inflammation could be the first step in infection-mediated preterm birth; as a consequence, the cervix shortens. A therapy that prevents cervical shortening would presumably be less effective if infection is already present. Ultimately, the safety and effectiveness of a therapy to prevent cervical dysfunction need to be addressed in a clinical trial.

There is a strong need in the field for new therapies to prevent preterm birth. Biocompatibility is a critical factor for success of an injectable therapy. This work provides support for the safety of silk gels in the cervix. Future studies will focus on how the silk gel changes the tissue properties in vivo and the effect of these changes on parturition. These studies are important steps toward developing a safe and effective alternative to cervical cerclage, which would be an exciting clinical development.

Authors' Note

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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