Soaking of Autologous Tendon Grafts in Vancomycin Before Implantation Does Not Lead to Tenocyte Cytotoxicity

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Background: Surgical site infections (SSIs) after anterior cruciate ligament (ACL) reconstruction procedures are an unfortunate complication. Soaking grafts in vancomycin before implantation has been reported to reduce the incidence of postoperative SSI after ACL reconstruction. There is potential for vancomycin to compromise graft integrity because of tenocyte toxicity.

Purpose: To examine the in vitro toxicity of varying doses of vancomycin on human tenocytes.

Study Design: Controlled laboratory study.

Methods: Human patellar tenocytes were isolated and expanded in vitro. Tenocytes in culture were exposed to vancomycin at 5 different concentrations (400, 1600, 3200, 6400, and 12,800 μg/mL) and 3 time intervals (2, 6, and 24 hours). The control for all series was tenocyte exposure to only culture medium for each time interval. After treatment, a 10% Cell Counting Kit-8 solution in cellular growth medium was applied to the cells to examine cytotoxicity. A live/dead assay was used to assess tenocyte viability through fluorescence microscopy and flow cytometry. Results were analyzed statistically using multivariable logistic regression models with Tukey honest significant difference post hoc tests.

Results: Vancomycin did not cause significant changes in tenocyte viability after 2 and 6 hours of incubation at any concentration between 0 and 12,800 μg/mL. Incubation with vancomycin for 24 hours led to a significant decrease in cell viability at higher concentrations.

Conclusion: Tenocytes derived from human patellar tendons exposed to relatively high concentrations of vancomycin for short periods of time do not demonstrate significant cell death and toxicity.

Clinical Relevance: Exposing tendons to vancomycin for a short period of time, such as before ACL reconstruction, is not likely to cause tenocyte toxicity because of vancomycin administration.

Keywords: tendon; tenocytes; toxicity; vancomycin; ACL

Surgical site infections (SSIs) are a rare but potentially catastrophic complication in orthopaedic surgery. Even with standardized preoperative protocols, SSIs still occur in up to 1% to 2% of all orthopaedic procedures.7,11 The rate of infection among sports medicine procedures is lower, but data still indicate a rate of 0.14% to 1.7% for anterior cruciate ligament (ACL) reconstructions in particular.2,6,13,15,20,21 SSIs not only contribute to patient morbidity but also lead to significantly increased costs in the forms of repeat surgeries, additional hospital stays, and increased overall health care utilization.5,23,27

Use of antibiotics, such as vancomycin, has been adopted by some surgeons as an attempt to reduce the incidence of SSIs. The high local bactericidal properties coupled with lower systemic toxicity make vancomycin a popular antibiotic choice.16,28 Recently, some have advocated for soaking ACL grafts in vancomycin solution before implantation, with most reports utilizing a concentration of 5 mg/mL.1,22,25,31 The practice of surgical field antibiotic use has historically been confined to spine procedures18,29,30 and joint arthroplasties,14,24,32 with the former demonstrating a reduced incidence of SSIs with application of topical vancomycin.8,10,28,30 These procedures, however, are qualitatively different from sports medicine procedures, particularly ACL reconstruction, where biological incorporation and toxicity to local tissues is more of a concern.

Little data exist regarding the toxicity of common antibiotics to tenocytes. The purpose of this study was to examine the in vitro toxicity of varying doses of vancomycin on human patellar tenocytes utilizing different exposure durations. This study’s hypothesis is that increasing dosage and exposure time to vancomycin would lead to increased tenocyte toxicity.
METHODS

Cell Isolation and Culture

Institutional review board approval and patient consent were obtained before conducting this investigation. Primary human tenocytes were explanted from patellar tendon tissue collected from a healthy human patient (age, 26 years) undergoing ACL reconstruction with bone–patellar tendon–bone autograft. To isolate human tenocytes, the collected tendon tissue was minced into small pieces (1 × 1 mm) in a petri dish and washed twice with phosphate-buffered saline (PBS; Gibco) and 1% penicillin-streptomycin (P/S; Gibco) under sterile conditions. The tissue was then placed in a standard 75-cm² tissue culture flask and cultured in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 (Gibco) supplemented with 20% fetal bovine serum (Gibco) and 1% P/S. The tendon tissue was incubated in humidified conditions at 37°C and 5% CO₂, with media changes every 2 to 3 days. After 10 days, cells began to emerge from the tendon tissue and started to adhere to the tissue culture flask. When tenocytes reached 80% to 90% confluency, they were treated with 0.05% trypsin–ethylene diamine tetra-actic acid (EDTA) and subcultured until passage 3, at which time the cells were seeded into 24-well plates at a density of 20,000 cells/cm² and cultured for 3 days before subsequent experiments.

Antibiotic Exposure

Tenocytes in culture were exposed to vancomycin at 5 different concentrations and 3 time intervals to mimic regional variations of topical administration. These concentrations were selected based on existing literature surrounding estimated intraoperative vancomycin concentrations as well as in vitro studies regarding the antibiotic’s effects on other human cells.9,16 Specifically, concentrations used to soak the ACL graft have ranged from 1 to 5 mg/mL.4,22,31 To create the vancomycin solution, lyophilized vancomycin hydrochloride was directly dissolved into the cell culture media and serially diluted to produce 5 different concentrations (400, 1600, 3200, 6400, and 12,800 µg/mL). Tenocytes were exposed to vancomycin for either 2, 6, or 24 hours. The control for all series was tenocyte exposure to only cul-

Cytotoxicity

After 2, 6, or 24 hours, the vancomycin was thoroughly washed out of each well with PBS. A 10% Cell Counting Kit-8 (CCK-8) solution (Abcam) in cellular growth medium was applied to the cells, followed by incubation for 2 hours at 37°C. The CCK-8 solution contains a water-soluble, 2-(2-methoxy-4-nitrophenoxy)-3-(4-nitrophenoxy)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt that is bioreduced in the presence of live cells to form an orange formazan dye, allowing for the quantification of cellular viability. The amount of formazan produced is directly proportional to the number of living cells. At each time point, tenocytes were incubated for 2 hours with the CCK-8 solution, and supernatant absorbance was measured at 450 nm using a spectrophotometer (SpectraMax M5; Molecular Devices) per the manufacturer’s protocol. Cell viability was expressed as a percentage of the control (untreated) cells.

Fluorescence Microscopy

After 2, 6, or 24 hours, the vancomycin was thoroughly washed out of each well with PBS. A LIVE/DEAD Cell Imaging Kit (Invitrogen) consisting of 1 µM calcein acetoxymethyl (AM) and 1 µM ethidium homodimer-1 was mixed 1:1 with culture media and added to each well to stain the cells for qualitative florescence imaging. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity as determined by the enzymatic conversion of the virtually nonfluorescent, cell-permeant calcein AM to the intensely fluorescent calcein, which is well-retained within live cells. The red component of the LIVE/DEAD Cell Imaging Kit is cell impermeant and therefore only enters cells with damaged membranes. In dying and dead cells, a bright red fluorescence is generated upon binding to DNA. Staining was performed per the manufacturer’s protocol. Tenocytes were visualized using a Keyence BZ-X700 fluorescence microscope, and images were taken at 10× magnification.

Flow Cytometry

Fluorescence-activated cell sorting (FACS) was used to quantify the number of live and dead cells at each concentration and time point. After 2, 6, or 24 hours, the antibiotic...
was thoroughly washed out of each well with PBS. Cells adherent to the culture wells were incubated with 0.25% trypsin-EDTA. After the tenocytes detached, trypsin-EDTA was neutralized with complete cell culture media, and cells and supernatant were centrifuged at 1200 rpm for 5 minutes at room temperature. The cells were then stained with the LIVE/DEAD Cell Imaging Kit, a sensitive 2-color fluorescence cell viability assay optimized for fluorescein isothiocyanate and Texas Red filters that allows discrimination between live and dead cells with 2 probes that measure recognized parameters of cytotoxicity and cell viability. The live cell component produces an intense, uniform green fluorescence in live cells (excitation/emission, 488 nm/515 nm). The dead cell component produces a predominantly nuclear red fluorescence (excitation/emission, 570 nm/602 nm). Based on the manufacturer's protocol, the live/dead kit was applied to each sample in equal volumes of 2 × working solution and incubated for 20 minutes at 25°C in the dark. After washing with flow wash buffer, samples were centrifuged at 1200 rpm for 5 minutes at room temperature and then resuspended with flow wash buffer. Live and dead cell counts were obtained by flow cytometric analysis (BD LSR Fortessa), and data were further analyzed using FlowJo software (version 10.6.2; Tree Star Inc).

Statistical Analysis

Data were collected in duplicate for all experiments and reported as mean ± SD. Multivariable logistic regression models with Tukey honest significant difference post hoc tests were used to analyze cell viability and cell death after vancomycin exposure. R software (version 3.6.1; R Foundation for Statistical Computing) was used for analysis, and an alpha level of .05 was set as significant.

RESULTS

Vancomycin did not cause significant changes in tenocyte viability after 2 and 6 hours of incubation at any concentration (Figure 1). Incubation with vancomycin for 24 hours led to a significant decrease in cell viability at higher concentrations, including 6400 μg/mL (P < .001) and 12,800 μg/mL (P < .001) (Figure 1). There was a significantly higher percentage of dead cells compared with the control after 24 hours of exposure to 12,800 μg/mL vancomycin (P < .001), but no difference for 400 μg/mL (P = .99) and 3200 μg/mL (P = .99) (Figures 2 and 3). The percentage of dead cells for the vancomycin groups did not change after 2 and 6 hours of exposure (Figure 3). Tenocyte morphology shifted from their typical elongated spindle-shaped appearance to become rounder at high concentrations of vancomycin (Figure 2).

DISCUSSION

The current study reports on the in vitro toxicity of vancomycin on human tenocytes. Our data demonstrated that vancomycin is not significantly toxic to tenocytes at concentrations up to 3200 μg/mL when cells are exposed for up to 24 hours. These concentrations are within the normally used concentration limits in clinical studies.

In tendon graft harvesting procedures, such as during ACL reconstruction, a potential infection may come from the graft harvest process or during graft preparation on the back table. Presoaking of ACL grafts in vancomycin has been reported in multiple studies as a way to decrease postoperative infection rates.22,32,35,51 Vertullo et al31 first described the technique of soaking ACL hamstring tendon autografts in vancomycin. The authors wrapped the harvested tendon in a Raytec swab that was presoaked in 5 mg/mL vancomycin. Out of 1135 patients whose grafts were soaked in vancomycin, zero infections occurred, compared with a 1.4% postoperative infection rate in patients whose grafts were not treated with vancomycin. Baron et al4 conducted a large, retrospective review of 1640 patients and reported that presoaking ACL grafts in vancomycin before ACL reconstruction resulted in a 10-fold decrease in postoperative infections. The autografts or allografts were soaked in 1 mg/mL vancomycin solution for an average of 10 minutes before reconstruction. Offerhaus et al22 analyzed postoperative infections after ACL reconstruction with autograft. The autografts were harvested from either the patellar, quadriceps, or hamstring tendons and were presoaked with 5 mg/mL vancomycin. Out of 853 patients, there were no postoperative infections, which was significant compared with the 1.4% infection rate seen in the control group where grafts were not presoaked. Additionally, their study found no difference in patient-reported outcomes between groups. Similarly, Schuster et al25 reported no postoperative infections in 2294 patients with ACL reconstruction after soaking hamstring tendon and quadriceps tendon grafts in

![Figure 1. Percentage viability after tenocyte exposure to 5 different concentrations of vancomycin for 2, 6, and 24 hours. Data are presented as mean ± SD. Statistical comparisons for each concentration were made against the control values (**P < .001).](image-url)
Figure 2. Live/dead fluorescence microscopy at 10 × magnification of human tenocytes after 2, 6, and 24 hours of exposure to 400, 3200, and 12,800 µg/mL vancomycin. Cells were stained with 1 µM calcein AM and 1 µM ethidium homodimer-1 to fluorescently label individual live (green) and dead (red) cells.

Figure 3. Fluorescence-activated cell sorting analysis of tenocytes after incubation with vancomycin at different concentrations and time points. Live cells were stained with fluorescein isothiocyanate (FITC) and dead cells with Texas Red (PE). Scatters show the distinguished live (lower box cluster) and dead (upper box cluster) cell populations.
5 mg/mL vancomycin before the reconstruction. The same group also found that soaking autografts in vancomycin was effective in reducing infections after revision ACL reconstructions. No infections occurred after 517 revision ACL cases performed with vancomycin-soaked grafts compared with an infection rate of 0.9% for the 1310 revision ACL cases without vancomycin.

The current investigation showed no significant toxicity in tenocytes exposed to vancomycin for 2 hours, even at concentrations upward of 12,800 µg/mL (12.8 mg/mL). Therefore, exposing tendon to lower concentrations up to 5 mg/mL vancomycin for a short, 10-minute duration may be reasonable to reduce postoperative infection rates after ACL reconstruction.

Previous in vitro studies on cellular toxicity of vancomycin have been conducted on relevant cell types within a joint. Bariteau et al found that exposing human mesenchymal stromal cells (hMSCs) to 5000 µg/mL vancomycin for 3 days resulted in significantly decreased cell viability, proliferation, and mineralization. Chu et al established a dose-dependent increase in cell death for hMSCs exposed to vancomycin for 24 hours, starting at 400 µg/mL. In the current investigation, tenocytes were exposed to the same concentrations of antibiotics as the above study, but there was no significant increase in cell death up to concentrations of 6400 µg/mL vancomycin. This finding suggests that various cell types respond differently to antibiotic exposure. Liu et al exposed osteoblasts, fibroblasts, and myoblasts to vancomycin for a short duration (1 hour) or continuous duration (48 hours). They found that for short durations, osteoblast and myoblast survival and migration were not affected by vancomycin, except for the highest concentration of 12 mg/mL. Similarly, in the current study it was found that a short, 2-hour exposure of vancomycin did not cause tenocyte toxicity.

In an in vitro study on the effects of vancomycin, tobramycin, and ciprofloxacin on osteoblasts and chondrocytes, it was found that vancomycin was the least toxic antibiotic, but vancomycin still caused significant decreases in skeletal cell proliferation at concentrations greater than 2000 µg/mL. These findings are applicable when considering soaking ACL grafts in vancomycin or for topical antibiotics in shoulder arthroplasty, where the rotator cuff tendons are bathed in antibiotic powder before skin closure.

In contrast to ACL graft soaking where the solution concentration can be closely controlled, there is no standard procedure for administering topical intrawound antibiotics during arthroplasty. Typically, 1 to 2 g of vancomycin powder is placed into the surgical wound before incision closure. Therefore, it is difficult to calculate exact in vivo concentrations of antibiotic, as the location and incision size vary between patients and contribute to differing doses. A limited number of studies have estimated in vivo concentrations of vancomycin based on postsurgical drain output, with concentrations between 263 and 2938 µg/mL in spine surgery and an average of 988 µg/mL (SD, 628) after total hip arthroplasty and 877 µg/mL (SD, 455) after total knee arthroplasty. Even at the highest initial concentration, these previously reported values are well below 6400 µg/mL, which was the concentration at which 24 hours of exposure to vancomycin started to cause toxicity toward tenocytes in our investigation. Multiple systematic reviews and meta-analyses, however, have concluded that intraoperative application of vancomycin powder is associated with a decreased incidence of SSIs after certain orthopaedic procedures. Combining this with the findings from the current investigation, we believe that the concentrations of vancomycin that have been used in clinical studies are safe with regard to tenocyte cytotoxicity.

There were several limitations to our investigation. As this was an in vitro study, 2-dimensional cell monolayers cannot fully model the in vivo environment of either tendon exposure to vancomycin powder in shoulder arthroplasty or presoaking of tendon autografts for ACL reconstruction. Further, in the joint, the multiple tissue types and extracellular matrix of tendons may protect tenocytes from toxic substances such as these high concentrations of antibiotics. While this study used tenocytes from the patellar tendon, there could also be biological differences in tenocytes isolated from different tendons, such as the hamstring. Additionally, tenocytes in the present study were exposed to a constant concentration of vancomycin throughout each time interval, but the vascularization and dynamic drug clearances in the body cause in vivo concentrations to decay exponentially over time. Also, cells in this study were exposed for up to 24 hours only and, because of this, could be overlooking longer-term metabolic or cytotoxic issues that may alter graft incorporation and healing. Finally, as tenocytes are adherent to cell culture flasks, dead cells that detached before staining were not able to be accounted for. As such, when performing FACS cell counting, the tenocytes that detached before staining were unable to be included in the cell death count. Therefore, the percentage of dead cells in each group may actually be higher than accounted for.

CONCLUSION

Tenocytes derived from human patellar tendons exposed to relatively high concentrations of vancomycin for short periods of time do not demonstrate significant cell death and toxicity.

REFERENCES


