

Efficacy of using a hydrodebrider and of citric acid/zwitterionic surfactant on a *Staphylococcus aureus* bacterial biofilm in the sheep model of rhinosinusitis

Rowan Valentine, M.B.B.S., Josh Jervis-Bardy, M.B.B.S., Alkis Psaltis, M.B.B.S., Ph.D., Lor Wai Tan, Ph.D., and Peter-John Wormald, M.D.

ABSTRACT

Background: Biofilms in chronic rhinosinusitis (CRS) patients are associated with recalcitrant disease patterns. Citric acid/zwitterionic surfactant (CAZS) has been shown to have significant efficacy at in vitro biofilm eradication. Unique hydrodebrider systems have been shown to have effective antibiofilm efficacy. The aim of this study was to evaluate the efficacy of the CAZS/hydrodebrider treatment on *Staphylococcus aureus* biofilms in the sheep model of CRS.

Methods: Forty-two sheep frontal sinuses were inoculated with *S. aureus*, followed by 7 days of biofilm growth. Sinuses were randomized to one of nine treatment groups: control, hydrodebrider with saline or CAZS (killed on day 0 or day 7 posttreatment), CAZS, or saline flush (killed on day 0 or day 7 posttreatment). Confocal scanning laser microscopy (CSLM) was used to confirm extent of biofilm reduction. Samples of each sinus were taken, assessing cilia morphology using electron microscopy.

Results: A mean of $37.22 \pm 9.65\%$ of control (no treatment) CSLM images taken showed biofilms. Both CAZS flush and CAZS/hydrodebrider treatments showed initial improvements in biofilms but biofilm regrowth followed treatment, $14.74 \pm 9.58\%$ to $18.91 \pm 12.14\%$ and $15.60 \pm 13.92\%$ to $24.70 \pm 3.66\%$, respectively ($p > 0.05$). Saline/hydrodebrider treatments showed a nonsignificant improvement in biofilm treatment, which was maintained, $18.34 \pm 11.85\%$ to $12.04 \pm 10.28\%$ ($p > 0.05$). Cilia morphology grade was significantly worse in the CAZS treatment groups ($p < 0.05$).

Conclusion: CAZS solution (with and without a hydrodebrider system) can significantly adversely affect cilia morphology. The hydrodebrider system, when combined with saline, may be a useful treatment adjunct for mucosal biofilms.

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Surgical intervention is warranted in medically recalcitrant chronic rhinosinusitis (CRS). The resistant nature of CRS to medical and surgical therapies has inspired ongoing research into the etiopathogenesis of CRS. It is estimated that at least 65% of all human bacterial infections involve biofilms.¹ Biofilms have been found to be important factors in the development of dental caries, periodontitis, otitis media, necrotizing fasciitis, biliary tract infections, osteomyelitis, bacterial prostatitis, native valve endocarditis, and cystic fibrosis pneumonia.^{2–4} The presence of bacterial biofilms in recalcitrant CRS patients has been well documented and has been shown to contribute to adverse effects on postoperative outcomes in CRS patients.⁵ Recent research has indicated that biofilms in CRS patients can be polymicrobial, with organisms such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and fungal biofilms being isolated. *S. aureus*, however, is the organism most frequently isolated from intraoperative culture and mucosal specimens, and its presence is associated with more severe and surgically recalcitrant disease patterns.⁶

Bacterial biofilms are defined as a structured community of bacterial cells enclosed in a self-produced extracellular polymeric substance (EPS) matrix and are adherent to an inert or living surface.⁷ The biofilm phenotype allows altered gene expression resulting in down-regulation of metabolic activity and suppression of growth rate. Additionally, the exopolymetric matrix protects bacterial biofilms. These genotypic and phenotypic characteristics allow biofilm-mediated diseases to be 100–1000 times more resistant to antibiotics than their

planktonic counterparts.⁸ Biofilms resistance to antibiotic and surgical therapies has led to a focus on mechanical disruption of bacterial biofilms. Effective biofilm removal has been hypothesized to include two main components: a chemical surfactant that interacts with the calcium-ion bridges of the biofilm and disrupts the EPS structure and a device that allows a shear force to be delivered to the mucosal surface. Recently, an agent that combines citric acid and zwitterionic surfactant (CAZS) has been developed. Citric acid combines with the calcium-ion bridges to disrupt the chemical binding of the EPS chains, and the zwitterionic surfactant dissolves the disconnected chains.⁹ CAZS has recently been shown to have significant efficacy at in vitro biofilm eradication capabilities.⁹ The recent development of a unique hydrodebrider endoscopic sinus irrigation system (Medtronic, Jacksonville, FL) allows for the controlled delivery of a shear force to the mucosal surface, and with the combination of CAZS solution may provide an effective treatment strategy for biofilms in CRS patients. The aim of this study was to evaluate the efficacy of the CAZS/hydrodebrider treatment on *S. aureus* biofilms in the sheep model of CRS.

METHODS

Approval for this blinded, prospective, randomized controlled trial was granted by the animal ethics committees of the University of Adelaide and the South Australian Institute of Medical and Veterinary Science.

Bacterial Inoculum

A well-documented biofilm-forming strain of *S. aureus*, reference strain ATCC 25923, was supplied by the Department of Microbiology at The Queen Elizabeth Hospital, Adelaide, South Australia, for use in this study. The *S. aureus* inoculum was prepared by subculturing it in anaerobic conditions overnight in Columbia Horse Blood Agar (Oxoid, Thebarton, South Australia) at 35.4°C. Single colonies of *S. aureus* were added to 5 mL of sterile 0.9% normal saline and diluted to a 0.5 McFarlane standard. The inoculum was then transferred to test tubes and placed on ice ready for instillation.

From the Department of Surgery–Otorhinolaryngology, Head and Neck Surgery University of Adelaide and Flinders University, Adelaide, Australia

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Address correspondence and reprint requests to Peter-John Wormald, M.D., Department of Otorhinolaryngology, Head and Neck Surgery, Queen Elizabeth Hospital, 28, Woodville Road, Woodville, SA 5011, Australia

E-mail address: peter.j.wormald@adelaide.edu.au

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Anesthetic

Twenty-one merino cross-wether sheep were used in this study, with all animals of 2–4 tooth-age and of similar size and weight. The sheep were transported from an animal holding facility 2 days before the surgical procedure to allow for acclimatization and fasted overnight. Each sheep received i.v. phenobarbitone (19 mg/kg) as an induction agent and maintenance anesthesia was maintained with 1.5–2% inhalational halothane. Each animal was placed in a supine position with the head supported in an upright plane using sandbags. The surgical field was shaved and prepared with half strength Betadine solution (Mundipharma Pharmaceuticals B.V., Amasfoot, The Netherlands). Sterile drapes were placed around its snout and torso. Three sprays (300 μ L) of topical Cophenycaine forte (ENT Technologies, Victoria, Australia) were used to decongest each nasal cavity 10 minutes before surgery. Intraoperative oximetry was used to monitor the sheep oxygen status throughout the surgical procedure.

Endoscopic Sinus Surgery

In vivo biofilm growth was performed as per the standardized operative technique described in a previous study by our department.¹⁰ Briefly, A middle turbinectomy was initially performed to allow access into the nasal cavity. Next, monopolar diathermy was used to ensure absolute hemostasis of the remnant edges. Removal of the anterior ethmoid terminus was then performed to allow endoscopic identification of the frontal sinus ostium and recess. All sheep were then extubated and returned to their individual pens. Sheep were monitored three times daily, with variables such as food intake, nasal discharge, and temperature observed. Subsequently, all animals were then transported to a holding facility to enable a recovery phase of at least 4 weeks duration, in accordance with animal ethics guidelines.

All sheep then returned for the inoculation surgery. All animals underwent general anesthesia as previously described. The frontal sinus was then trephinated at the site 1 cm lateral to the sagittal plane, along a line marked between the midpoints of each bony orbit. The frontal sinus ostia and recess was confirmed by the instillation of fluorescein (0.1 mL diluted in 10 mL of normal saline). Each sinus was flushed with 50 mL of sterile saline through the minitrephine. Two 30-cm-long sterile Vaseline-coated gauze ribbons (Covidien, Mansfield, MA) were then carefully packed into the frontal recess to ensure a water-tight seal of the frontal sinus. Resistance encountered on attempted flushing of the frontal sinus provided confirmation of the sinus obstruction/seal. The frontal sinus was then inoculated with 1 mL of the prepared dilution of *S. aureus* via the minitrephines. The minitrephines were securely capped to prevent any external leakage of bacteria. Postoperatively, the sheep were then extubated and monitored as previously described. Biofilms were allowed to grow for 7 days according to previous *in vitro* data.¹¹

Treatment Groups

Preoperative sinus treatment randomization was performed, and after the initial 7 days of bacterial inoculation each frontal sinus was treated accordingly. Four sinuses were randomized to one of eight treatments. Treatment for all arms was applied while under general anesthesia as previously described. Each treatment began with reventilation of the frontal sinus by complete removal of the Vaseline-coated gauze. The hydrodebrider treatments involved the use of an electronically activated instrument connected to a mechanically driven pump that delivers the irrigant under high pulsatile pressure through a stainless steel arm measuring 19 cm in length and 2 mm in diameter, to a bent distal tip that contains a rotating nozzle designed to produce a target spray angle of 30° in one direction. The distally controlled rotating nozzle allowed an effective spraying angle of 60° within the frontal sinus cavity. The hydrodebrider was placed into the sinus cavity endonasally and operated in the "ON" mode for 3

seconds. The sinus cavity was then cleared with suction before the next 3-second interval, with the cycle being repeated five times. The spray tip is then rotated 90° and the cycle was repeated until 360° of rotation had been completed. The sinus cavity was flushed with 50 mL of the irrigant solution at the completion of treatment. Total volume used of irrigant were ~500 mL/sinus.

No Treatment (Maximal Biofilm Growth). Four sinuses did not receive any treatment and the sheep were killed 7 days after initial sinus inoculation.

Hydrodebrider with Saline (Killed on Day 0 Posttreatment). These sinuses were treated with the use of the hydrodebrider with saline as the irrigant. These sheep were then killed immediately after treatment.

Hydrodebrider with Saline (killed on Day 7 Posttreatment). The sinuses were treated as described previously for the hydrodebrider use with saline as the irrigant. These sheep were killed on day 7 posttreatment.

Hydrodebrider with CAZS (Killed on Day 0 Posttreatment). The sinuses were treated as described previously for the hydrodebrider use with CAZS solution as the irrigant. These sheep were killed on day 0 posttreatment.

Hydrodebrider with CAZS (Killed on Day 7 Posttreatment). The sinuses were treated as described previously for the hydrodebrider use with CAZS solution as the irrigant. These sheep were killed on day 7 posttreatment.

Saline Flush (Killed on Day 0 Posttreatment). The sinuses were treated with 500 mL of syringe flush with sterile saline via the minitrephine. These sheep were killed on day 0 posttreatment.

Saline Flush (Killed on Day 7 Posttreatment). The sinuses were treated with 500 mL of syringe flush with sterile saline via the minitrephine. These sheep were killed on day 7 posttreatment.

CAZS Flush (Killed on Day 0 Posttreatment). The sinuses were treated with 500 mL of syringe flush with CAZS solution via the minitrephine. These sheep were killed on day 0 posttreatment.

CAZS Flush (Killed on Day 7 Posttreatment). The sinuses were treated with 500 mL of syringe flush with CAZS solution via the minitrephine. These sheep were killed on day 7 posttreatment.

Frontal Sinus Mucosa Harvest

Mucosal specimens from the frontal sinus were collected for tissue analysis on day 0 or day 7 posttreatment. The sheep were killed and a skin flap overlying the frontal bone was raised using a sterile scalpel blade and periosteal elevator. A 2 × 2-cm bony window was created using a drill equipped with disposable sterile rotatory steel-cutting blades (Dremel, Sydney, Australia) to access the frontal sinus. The mucosal specimens were carefully lifted off the periosteal wall, removed *en bloc*, and immediately placed in Dulbecco's modified eagle's media (Sigma Aldrich, NSW, Australia).

Quantification of Biofilm Surface Area Coverage Using Peptide Nucleic Acid Fluorescence *In situ* Hybridization Assay

A mucosal sample measuring 1 cm² was excised for fluorescence *in situ* hybridization (FISH) immediately after tissue harvest. Biofilm bacteria adherent on the mucosal surface were fluorescently labeled using a commercially available *S. aureus* PNA FISH kit (AdvanDx, Woburn, MA). Hybridization of an Alexa 488-labeled peptide nucleic acid probe to a species-specific 16S ribosomal sequence of *S. aureus* was performed. One drop of the fixation solution (phosphate-buffered saline [PBS] with detergent) and 20 minutes of heating at 55°C was used to fix each specimen. Coated microscope slides were used (SnowCoat; Surgipath, Richmond, IL). The extent of biofilm growth was determined by the number of slides with mucosal involvement using confocal scanning laser microscopy (CSLM). The slides were then immersed in 90% ethanol for 10 minutes and allowed to air-dry for 10 minutes. One drop of the Alexa 488 *S. aureus* FISH probe was

Table 1 Frequencies of cilia morphology grades

Treatment		Grade 1	Grade 2	Grade 3	Grade 4
Control		0	11	5	0
CAZS flush	Day 0	2	12	2	0
Saline flush	Day 0	0	14	2	0
Hydrodebrider/CAZS	Day 0	0	13	3	0
Hydrodebrider/saline	Day 0	3	9	3	1
CAZS flush*	Day 7	0	5	8	3
Saline flush	Day 7	0	14	2	0
Hydrodebrider/CAZS#	Day 7	0	3	9	4
Hydrodebrider/saline	Day 7	0	14	2	0

* $p = 0.05$; # $p = 0.007$.

used per sample. A coverslip was placed on the mucosal surface and the slides were placed into a humidified chamber and incubated in darkness at 55°C for 90 minutes. The coverslips were removed and the slides underwent a stringent wash procedure with preheated wash solution (Tris-buffered saline with detergent) at 55°C for 30 minutes. Air-drying was then performed for 10 minutes followed by slide transportation to Adelaide Microscopy for analysis by CSLM. Mounting medium was then applied to the slides (1 drop) followed by a thin coverslip. The presence of green microcolonies (with surrounding green haze) at the excitation wavelength of 488 nm was determined to be biofilm. An appropriate z-level that was determined to be at the mucosal plane of tissue was selected and fixed. The software available on the Leica Application Suite—Advanced Fluorescence (Leica Microsystems, Wetzlar, Germany)—allowed the generation of 100 fields of view, at 20× magnification, of random sites across the entire surface of the mucosal specimen. The offset and gain settings were kept fixed at −0.3 and 850%, respectively. Two blinded observers each manually analyzed for the presence or absence of mucosal surface and the presence or absence of biofilm. Because of the three-dimensional nature of the mucosal surface, some slides failed to scan at the mucosal surface and were therefore excluded. The sum of all slides with biofilm involvement was accrued and compared with the total number of slides taken at the mucosal surface to determine an average percentage of slides taken with biofilm involvement on the mucosal surface.

Cilia Morphology

Specimens for scanning electron microscopy were placed in PBS and then washed for 20 minutes using an ultrasonic cleaner to remove blood clots, mucous, debris, and biofilm. The specimens were then fixed in a solution of 4% paraformaldehyde/1.25% glutaraldehyde in PBS with 4% sucrose, pH 7.2. Five-millimeter-squared specimens were randomly chosen, and after three PBS washes samples were postfixed with 2% osmium tetroxide for 1 hour on a rotator. The samples were then dehydrated through a graded series of 70–100% ethanol. The samples were then critical-point dried in CO₂, mounted on scanning electron microscopy stubs, sputter coated with gold palladium, and examined using a Field Emission Gun Scanning Electron Microscope (XL30; Philips, Eindhoven, The Netherlands). Each surface image was graded according to a previously published grading scale (Table 1). Four pictures for each specimen, and taken at random (at 2000× magnification), were used to calculate the cilia grade.

RESULTS

Treatment

Table 2 summarizes our findings. A total mean of $37.22 \pm 9.65\%$ of control (no treatment) CSLM images taken showed biofilms. When

considered as a group, treatment of the sinuses showed a statistically significant reduction in biofilm growth when compared with no treatment at all ($p = 0.02$).

Irrigant Flush. All irrigant flush treatment sheep showed a reduction in the biofilm growth when compared with the control sinuses; however, this was nonsignificant. Eradication of biofilm was most effective in the sinuses irrigated with CAZS solution when compared with saline (mean, $14.74 \pm 9.58\%$ versus $21.39 \pm 12.51\%$, respectively; $p > 0.05$). However, only the CAZS treatment showed a biofilm regrowth 7 days after treatment, increasing from a mean of $14.74 \pm 9.58\%$ to $18.91 \pm 12.14\%$ ($p > 0.05$). Although CAZS solution was immediately effective at reducing the biofilm load, this effect was not maintained over time. The saline flush treatment failed to show a regrowth of biofilm and stayed relatively stable, from a mean of $21.39 \pm 12.51\%$ to $20.65 \pm 18.16\%$.

Hydrodebrider Treatment

All hydrodebrider-treated sheep showed a nonsignificant reduction in biofilm load, with the CAZS/hydrodebrider treatment outperforming the saline/hydrodebrider treatment (mean, $15.60 \pm 13.92\%$ versus $18.34 \pm 11.85\%$, respectively; $p > 0.05$). However, with regard to biofilm regrowth 7 days after treatment an interesting trend was noted. The CAZS/hydrodebrider treatment showed a biofilm regrowth (7 days after treatment) from a mean of $15.60 \pm 13.92\%$ to $24.70 \pm 3.66\%$, the highest regrowth of any treatment arm. This compares with the saline/hydrodebrider arm that showed a further nonsignificant reduction in biofilm presence after 7 days of observation posttreatment (from a mean of $18.34 \pm 11.85\%$ to $12.04 \pm 10.28\%$).

Cilia Morphology

Table 3 summarizes these results. Cilia morphology deteriorated after exposure to the CAZS solution. At day 0 there was no significant interaction between cilia grade and treatments when compared with control. However, at day 7 posttreatment a significant finding was noted. CAZS/hydrodebrider treatment significantly deteriorated cilia grade 8 days after treatment when compared with control ($p = 0.007$). Additionally, 7 days after treatment, CAZS flush showed significant deterioration of cilia grade when compared with control ($p = 0.05$). CAZS flush-treated sinuses deteriorated (nonsignificantly) from a mean grade of 2.0 immediately after treatment to 2.875 7 days posttreatment. This finding was also noted in the CAZS/hydrodebrider treatment with deterioration from 2.188 to 3.063 7 days posttreatment. This observation was not shown in the saline flush or saline/hydrodebrider treated sinuses. Both the saline flush and the saline/hydrodebrider treatments maintain cilia morphology over time, at a stable mean value of 2.125 for both of these treatments. Although this data are nonsignificant, it implies that cilia morphology may be unaffected by the hydrodebrider system and that cilia toxicity is an observed effect of the CAZS solution.

DISCUSSION

All treatments established a biofilm clearance effect. CAZS treatment, however, showed a biofilm regrowth effect and concomitant cilia toxicity. The hydrodebrider, however, showed effectivity that was maintained to the 7-day assessment period. Several topically deliverable techniques have been investigated for the treatment of bacterial biofilms in CRS including baby shampoo, antibiotic and antifungal therapies, and even Manuka honey. With the improved understanding of the molecular and chemical mechanism that allow the extracellular polymeric matrix to provide protection to embedded organisms we can design targeted treatment strategies. Recently, there have been two studies investigating the *in vitro* antibiofilm and *in vivo* cilia effects of CAZS, showing its efficacy and safety profile.^{9,12} This is the first study to investigate the effect of CAZS with and without the use of a hydrodebrider system in an *in vivo* bacterial

Table 2 Summary of percentage of biofilm-positive slides analyses

Treatment	CAZS Flush	Hydrodebrider/CAZS	Saline Flush	Hydrodebrider/Saline
Day 0	14.7	15.6	21.4	18.3
Day 7	18.9	24.7	20.7	12.0
Day 0 vs day 7 significance	$p = 0.61$	$p = 0.29$	$p = 0.95$	$p = 0.45$
vs Control significance	$p = 0.09$	$p = 0.09$	$p = 0.09$	$p = 0.09$

Table 3 Cilia grade scores

Grade	SEM Appearance
I	Normal cilia with normal orientation
II	Ciliated epithelium but disoriented
III	Stumps of cilia and regenerating cilia
IV	No identifiable cilia

SEM = scanning electron microscopy.

biofilm model. This study shows that although CAZS irrigation (with and without a hydrodebrider system) has an antibiofilm activity, it can significantly adversely affect cilia morphology. Adverse cilia morphology may result in a corresponding biofilm regrowth phenomenon. Although nonsignificant, saline irrigation combined with the hydrodebrider system was the most effective at reducing the mucosal biofilm load and preventing biofilm regrowth.

Tamashiro *et al.*¹² also investigated the effect of CAZS on sinonasal epithelium. This study showed that a single episode of CAZS irrigation in the healthy rabbit model had significant temporary adverse effects on cilia morphology. These effects, however, were noted to be temporary in nature with a return back to near normal cilia morphology 1 week posttreatment.¹² Although we did not establish any significant improvement in cilia morphology at this time point, it is important to consider the additional impact that mucosal biofilms have on ciliated sinonasal epithelium. Schipor *et al.*¹³ investigated the effects of biofilm forming *P. aeruginosa* on ciliary function. Results showed that wild-type *P. aeruginosa*-infected mucosa showed massive ciliary damage and concomitant absence of beating cilia. Our study has used the diseased sheep model of bacterial biofilms. Although there is an initial effect of biofilm treatment, there was a trend toward regrowth of the biofilms 1 week posttreatment. With a significant effect of CAZS/hydrodebrider on the deterioration of cilia morphology, this regrowth phenomenon may be caused by disruption of the normal mucociliary clearance of bacteria from the sinonasal epithelium.

Hydrodebrider systems have had extensive success in the orthopedic literature as an effective method of removing biofilms from open wounds and prosthetic devices.¹⁴ The use of powered irrigation techniques has been shown to increase the removal of bacteria by a 100-fold.¹⁴ This mechanical disruption effect has also been seen in both the *in vitro* and the *in vivo* scenario.^{9,15} Le *et al.*¹⁵ also indicated that in the same *in vivo* setting the CAZS/hydrodebrider treatment also showed significant antibiofilm efficacy; however, regrowth of biofilms were also noted.¹⁵ Although only a pilot study, we have showed that the hydrodebrider system, when combined with saline irrigant, trended toward having the greatest lasting effect on mucosal biofilm load, likely because of the mechanical disruption caused on the mucosal biofilm EPS matrix. It is important to note that the saline irrigation treatment, in combination with the hydrodebrider, showed no significant difference in cilia morphology. This mechanical disruption effect may have allowed the innate immune system and mucociliary clearance to "mop up" the fragmented biofilm load.

It is important to be aware of some potential limitations to this

study. Because only a small portion of the frontal sinus mucosa could be analyzed, there is a potential for a sampling error. It was difficult to ensure that the hydrodebrider made direct contact with all areas of the sinus mucosa due to the large amount of sinus septations. Additionally, this study was a pilot study only with small numbers in each treatment arm. Although cilia morphology is an important component to cilia function, we did not analyze cilia function using cilia beat frequency testing.

CONCLUSION

The presence of bacterial biofilms in recalcitrant CRS patients has been well documented. This study indicates that although CAZS irrigation (with and without a hydrodebrider system) has an antibiofilm activity, it can significantly adversely affect cilia morphology. Impaired cilia morphology seems to lead to biofilm regrowth. We have shown that the hydrodebrider system, when combined with saline irrigant, may be a useful treatment adjunct for mucosal biofilms and has no significant adverse effect on cilia morphology.

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