



Efficacy of anti-biofilm gel, chitogel-mupirocin-budesonide in a sheep sinusitis model

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Background: The search for effective topical anti-inflammatory and antibiofilm delivery to manage recalcitrant chronic rhinosinusitis (CRS) continues to be elusive. The ideal topical treatment aims to provide adequate contact time for treatment agents to exert its effect on sinus mucosa. Our study presents the *in vivo* efficacy of chitogel (CG), combined with anti-inflammatory agent budesonide and antibiofilm agent mupirocin (CG-BM) for treatment of *S. aureus* biofilms.

Methods: In established sheep sinusitis model, 15 sheep were randomised into three groups (5 sheep, n=10 sinuses, per treatment): (I) twice daily saline flush (NT), (II) CG with twice daily saline flush, and (III) CG-BM with twice daily saline flush for 7-days. The anti-inflammatory effect was graded by an independent blinded pathologist. The antibiofilm effect was assessed using LIVE/DEAD BacLight stain and confocal scanning laser microscopy.

Results: No significant differences were observed in histopathology between all groups. A significant reduction in biofilm biomass was shown using COMSTAT2 in CG-BM treated sheep compared to NT controls ($P < 0.05$, One-way ANOVA, Kruskal-Wallis test).

Conclusions: CG-BM is effective against *S. aureus* biofilms in a sheep sinusitis model and could represent a viable treatment option in the clinical setting for recalcitrant CRS.

Keywords: Chronic rhinosinusitis (CRS); *Staphylococcus aureus*; biofilm; chitogel (CG); budesonide; mupirocin; topical; gel; antimicrobial therapy

Received: 04 April 2019; Accepted: 27 March 2020; Published: 12 May 2020

doi: 10.21037/ajo-18-97

View this article at: <http://dx.doi.org/10.21037/ajo-18-97>

Introduction

Recalcitrant chronic rhinosinusitis (CRS) has been increasingly recognised to be attributed to bacterial biofilms (1-5). Amongst surgically recalcitrant patients about 40–50% of biofilms identified are *Staphylococcus aureus* (6,7). Oral antibiotics are often ineffective against biofilms (8), pressuring a continuous search for topical anti-biofilm

agents which allows for increased concentration, localised action and less systemic side effects.

The effective delivery of these topical treatments into sinus cavities remains the main obstacle. Currently topical antibiotics are administered via sinonasal irrigations, nebulisers and nasal sprays. All of these methods lack the ideal characteristics of complete sinus distribution, prolonged mucosal contact time to increase local absorption

and minimal waste (9). Patients receive highly variable drug penetration influenced by patient condition, sinus surgery, delivery devices, irrigation volume and pressure, and patient positioning (9-12). To overcome all these challenges, we looked at investigating the efficacy of CG (chitosan and dextran), a surgical hydrogel FDA approved for the use after sinus surgery, acting as a drug carrier to deliver the anti-inflammatory effects of budesonide and antibiofilm effects of mupirocin in a previously validated *S. aureus* biofilm sheep sinusitis model (13,14).

The aim of this study was to (I) optimize a sheep sinusitis model for the investigation of a topical gel treatment and (II) to evaluate the antibiofilm and anti-inflammatory effects of CG-budesonide-mupirocin (CG-BM) in the treatment of *S. aureus* biofilms *in vivo*.

Methods

Ethics approval was obtained from the Animal Ethics Committee of The University of Adelaide and the South Australian Health and Medical Research Institute (SAHMRI). All reports on animal experiments have been conducted in accordance with institutional and national guidelines for the care and use of laboratory animals.

Optimisation arm

A total of 5 male merino sheep heads were obtained from the Murray Bridge Abattoir, South Australia. To achieve optimum retention of the gel within the frontal sinuses for 5–7 days, we optimised the method of gel application and determined the appropriate volume of saline flush to be 15 mL twice a day commencing 24 hours after gel instillation.

Study arm

Animals

A total of 15 male merino sheep between the dental age of 2 to 4 years were used. All animals were drenched to eradicate the parasite *Oestrus Ovis*.

Bacterial inoculum

Reference strain American Type Culture Collection (ATCC) 25923 *Staphylococcus aureus*, known to be biofilm forming, was supplied by the Queen Elizabeth Hospital Department of Microbiology, Adelaide. Frozen glycerol stock was defrosted and subcultured for 24 hours in 3mL of nutrient broth (Oxoid, Adelaide, Australia) on a shaker at 37 °C

before inoculum was transferred to a 1% nutrient agar plate (Oxoid, Adelaide, Australia). The plate was incubated for 16–18 hours at 37 °C and a single colony forming unit (CFU) was diluted to 0.5 McFarland standard in 0.45% sterile saline and transferred on ice for instillation into sheep sinuses.

CG

CG comprised of three components; 5% succinyl-chitosan, 0.3% phosphate buffer and 3% dextran aldehyde (Chitogel®, Wellington). All components were manufactured and sterilised by Chitogel® and cultured for sterility by the Department of Microbiology, Princess Margaret Hospital, Western Australia prior to being used in this study. All stocks were stored at room temperature.

Preparation of CG

Dextran aldehyde was first dissolved in 10 mL of phosphate buffer then mixed with 10 mL of succinyl-chitosan using sterile technique.

Preparation of CG-BM

Ten mg of powdered mupirocin (PCCA, Houston) was solubilised under sterile conditions in 6 mL of phosphate buffer 24 hours before application. Mupirocin solution was mixed with 2 mg/4 mL of Pulmicort respules (AstraZeneca Ab, Sodertalje) before being used to dissolve dextran aldehyde and then mixed with 10mL of succinyl-chitosan using sterile technique.

Anaesthetic protocol

All sheep were fasted for 12 hours prior to general anaesthesia. They were induced with intravenous phenobarbitone (19 mg/kg), intubated and placed onto 1.5% to 2% inhalation isoflurane over the course of the procedure. All sheep were placed supine on a wooden cradle and supported with neck slightly flexed on a head ring. Two sprays of Cophenylcaine Forte (ENT Technologies Pty Ltd, Australia) were applied to each nasal cavity 10 minutes prior to procedure.

Surgical protocol

As per protocol (13,14) all sheep had middle turbinectomy and anterior ethmoid complex resection followed by a 3–4 week recovery. Then frontal mini-trephines were placed bilaterally on the sheep's forehead, 1cm lateral from the midline at the level of superior orbital rims. Accurate trephine placements were verified when fluorescein flushed

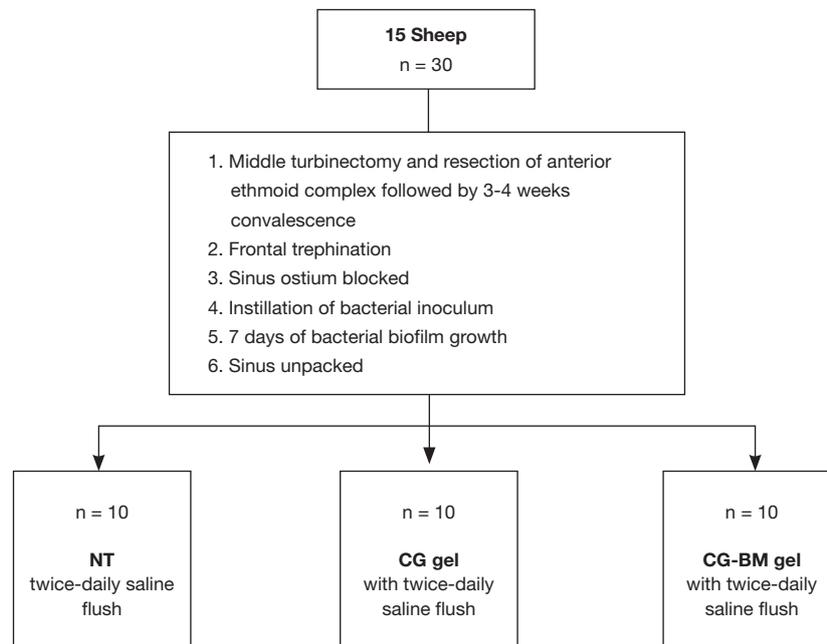


Figure 1 Flow diagram illustrating protocol and efficacy groups. Each sheep was randomised into: (I) twice daily saline flush (NT), (II) CG with twice-daily saline flush, and (III) CG-BM with twice-daily saline flush. NT, no treatment; CG, chitogel; CG-BM, chitogel-budesonide-mupirocin.

via trephines (0.1 mL diluted in 100 mL of normal saline) can be visualised endoscopically draining from the frontal sinus ostium into nasal cavity.

Efficacy arm

After frontal trephination, petroleum gauze (Vaseline, Kendall, Mansfield, MA, USA) were used to pack the frontal ostiums. One mL of 0.5 McFarland Units *S. aureus* was instilled via mini-trephines into each sinus cavity and biofilms were allowed to form over 7 days. On day 8, the petroleum gauze was unpacked and each sheep was randomly assigned into one of three efficacy groups: (I) twice-daily NT, (II) CG and (III) CG-BM. For sheep assigned to CG and CG-BM groups, the gels were instilled once via mini-trephines into each sinus cavity until extrusion visualised under direct endoscopic view from the frontal sinus ostium. All sheep received sinus irrigations with 15 mL of sterile normal saline twice a day commencing 24 hours after gel application for a total of 6 days. Sinus irrigations were commenced 24 hours later based on protocol of previous clinical studies (15,16). All sheep were euthanised on day 8 and sinus mucosa harvested for histopathological analysis and biofilm biomass imaging

(Figure 1).

Biofilm imaging

From each sinus cavity, two random sections of 1 cm x 1 cm sinus mucosa were sampled and briefly immersed in phosphate buffered solution to wash off planktonic cells. Sampled mucosa was then stained with LIVE/DEAD BacLight stain (Life Technology, Mulgrave, Victoria, Australia) as per manufacturer’s instructions. Confocal scanning laser microscope (Zeiss Germany) were used to assess biofilm biomass. Three Z-stack images of highest biofilm presence were taken of each sample (Image properties: line average 4, 512x512 pixels, Z-stack 80 steps) making a total of 6 Z-stack images per sinus. COMSTAT2 software (Lyngby, Denmark) was later utilised to quantify biofilm biomass from each Z-stack.

Histopathology evaluation

From each sinus cavity, one 1 cm x 1 cm sinus mucosal section was fixed in 2% formalin solution and sent for histopathology analysis (Adelaide Pathology and Partners, Adelaide, Australia). Samples were stained with

Table 1 Histopathology Likert scale grading performed by a blinded independent pathologist comparing sheep sinus mucosa treated with (A) NT, (B) CG and (C) CG-BM looking at degree of acute inflammation, oedema and fibrosis

| Variable | Acute inflammation (0–2) | Oedema (0–3) | Fibrosis (0–3) |
|------------|--------------------------|--------------|----------------|
| NT | | | |
| M6 left | 1 | 2 | 0 |
| M6 right | 1 | 2 | 1 |
| M7 left | 1 | 2 | 0 |
| M7 right | 2 | 2 | 0 |
| M11 left | 1 | 2 | 1 |
| M11 right | 1 | 2 | 0 |
| M12 left | 1 | 2 | 0 |
| M12 right | 1 | 2 | 0 |
| M13 left | 1 | 2 | 0 |
| M13 right | 1 | 2 | 0 |
| CG | | | |
| M1 left | 1 | 2 | 0 |
| M1 right | 1 | 1 | 0 |
| M3 left | 1 | 2 | 0 |
| M3 right | 2 | 2 | 0 |
| M5 left | 1 | 2 | 1 |
| M5 right | 1 | 2 | 1 |
| M8 left | 1 | 2 | 1 |
| M8 right | 1 | 2 | 0 |
| M14 left | 1 | 2 | 0 |
| M 14 right | 1 | 2 | 0 |
| CG-BM | | | |
| M2 left | 1 | 2 | 0 |
| M2 right | 0 | 2 | 0 |
| M4 left | 2 | 2 | 1 |
| M4 right | 1 | 1 | 0 |
| M9 left | 1 | 1 | 0 |
| M9 right | 1 | 1 | 0 |
| M10 left | 1 | 2 | 0 |
| M10 right | 1 | 2 | 0 |
| M15 left | 1 | 2 | 0 |
| M15 right | 1 | 2 | 0 |

NT, no treatment; CG, chitogel; CG-BM, chitogel-budesonide-mupirocin.

hematoxylin & eosin and embedded in paraffin. Using light microscopy (Eclipse 90i, Nikon instruments Inc, Melville, NY, USA) a blinded pathologist performed microscopic evaluation and tissue grading. A Likert scale was used to grade, acute inflammation, oedema and fibrosis (*Table 1*).

Statistical analysis

Previous studies have described a 60% biofilm biomass reduction with treatment (14). To obtain a power of 80% and achieve a significance level of $\alpha=0.05$ four sheep were required per arm. Five animals per arm were used in this study as this was our first experience investigating the efficacy of a topical gel treatment.

Bacterial biofilms were compared between all groups and analysed with 1-way analysis of variance (ANOVA) Dunnett's multiple comparison test using GraphPad Prism 8.0 software (San Diego, CA, USA).

Results

Anti-inflammatory effects

There were no significant differences in acute inflammation, oedema and fibrosis between sinus mucosa treated with NT and CG-BM (*Table 1, Figure 2*).

Antibiofilm effects

There was a significant reduction in biofilm biomass of CG-BM treated sheep compared to NT controls [$P=0.01$, 4.74 (95% CI: 0.85 to 8.6)]. There were no significant difference between NT and CG treated sheep or CG and CG-BM treated sheep (*Figure 3*).

Discussion

In this study we demonstrated that CG-BM significantly reduced *S. aureus* biofilms in an *in vivo* sinusitis model. Compared to no-treatment controls, CG-BM and CG reduced *S. aureus* biofilms by 90.5% and 20% respectively.

CG comprises succinyl-chitosan which is a chitosan polymer produced by the hydrolysis of chitin, found in the exoskeletons of crustaceans. In the last decade, CG has been largely used in ENT surgery to improve patient's outcome post endoscopic sinus surgery (16-19) due to its effective hemostatic (18-26), wound healing (27-29), anti-

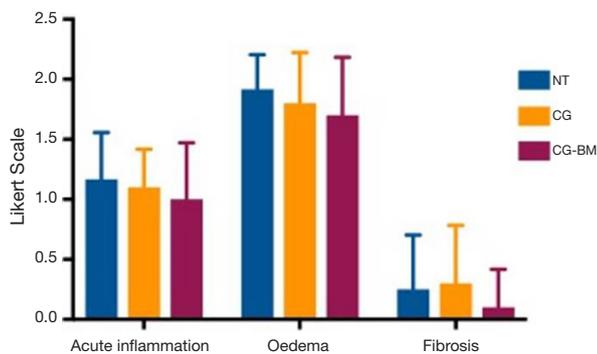


Figure 2 Bar graph showing no difference in histopathology assessment of sinus mucosa, specifically there were no anti-inflammatory effects observed, between NT and CG-BM treated group. NT, no treatment; CG, chitogel; CG-BM, chitogel-budesonide-mupirocin.

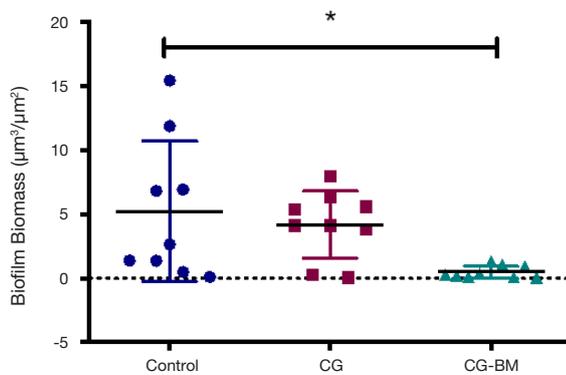


Figure 3 Significant reduction of biofilm biomass seen in CG-BM treated group compared to NT and CG. One-way ANOVA, Dunnett’s multiple comparison test. *, P<0.05. NT, no treatment; CG, chitogel; CG-BM, chitogel-budesonide-mupirocin.

adhesion (17,30-38) and antimicrobial (39-41) properties. However, its role as a drug release vehicle delivering topical treatments into sinus cavities has yet to be explored.

We hypothesize that by incorporating topical antibiotics into CG we are able to eradicate biofilms by direct application of antibiotic gel into sinuses, increase mucosal contact time with topical agents and use higher concentrations of antibiotics at target sites with less systemic side effects. Previous studies have also suggested that chitosan enhances the nasal absorption of topical treatments by the bioadhesivity of the polymer to mucosa and a transient widening of the nasal mucosa tight junctions (42,43).

Mupirocin was chosen to be the antimicrobial of choice

to be incorporated into CG due to its known clinical efficacy as a nasal irrigation for patients with recalcitrant *S. aureus* CRS (44-48). Jarvis-Bardy et al reported 88.9% of surgically recalcitrant patients had eradication of *S. aureus* using twice daily nasal lavages containing 0.05% mupirocin for 3 weeks (48). Mupirocin exerts its antimicrobial activity by irreversibly binding to the bacterial enzyme isoleucyl-transfer RNA, thereby preventing isoleucine incorporation during bacterial synthesis (49-51). It has excellent activity against staphylococci including MRSA, most streptococci, and against certain gram negative bacteria including *Haemophilus influenzae* (50) which are increasingly prevalent in clinical practice.

Budesonide irrigations are now widely recognised to be safe (52-54) and effective in improving symptoms and endoscopic outcomes post sinus surgery (55-57). Luo *et al.* suggested that hydrophobic drugs like steroids are released more slowly from hydrogels (58), making CG an excellent controlled release vehicle for budesonide. Our department has recently demonstrated that by incorporating budesonide in CG applied post sinus surgery, there was endoscopic evidence of reduced inflammation during the early postoperative period and reduction in the extent of ostial stenosis at 3 and 12 months (15) suggesting possible anti-inflammatory and anti-adhesion properties. CG-BM treated mucosa showed a trend to improvement in acute inflammation, oedema and fibrosis when compared to saline rinses, but was not statistically significant. Therefore we did not further our investigation on anti-inflammatory effects of individual gel components

We postulate that this could be a result of several different factors. Firstly, only intermediate levels of inflammation were observed in our positive control sheep, indicating that *S. aureus* biofilms might not induce massive inflammation in sheep as would normally be expected. This could be due to the fact that sheep sinus mucosa is naturally colonised by a range of bacteria and might be adapted to the presence of biofilms already. Secondly, the release of budesonide from CG was found to be only up to 18% of the total possible amount over 72 hours (unpublished observations). Optimising the pharmacological formulation of budesonide for improved release from CG might help to improve the anti-inflammatory effects of CG-Bud gel. Also, it is possible that the self-limiting inflammatory process post-surgery is different to inflammation secondary to a bacterial nidus, which might require a higher concentration or longer exposure of budesonide for an increased effect.

CG-BM gel can be applied in the outpatient setting

under direct endoscopic view into post-ESS infected sinus cavities via curved and straight suction cannulas. This has the theoretical benefit of effective anti-biofilm delivery specific to infected sinuses regardless of ostium size, patient douching technique and compliance. However further clinical studies are required to explore this treatment viability.

This study has several limitations, one such limitation was our inability to quantify the amount of treatment gel remaining in each sinus over the treatment course due to differing sinus anatomy or drainage. Therefore, it is possible that each sinus was exposed to variable drug concentrations during treatment. Another limitation of this study remains that *in vivo* studies cannot fully simulate the conditions of human sinuses and clinical studies will be required to fully characterise the potential of this treatment.

Conclusions

Our study concludes that CG-BM significantly reduces *S. aureus* biofilms in a sheep sinusitis model. The use of CG as a means to deliver topical therapies offers otolaryngologists a potential alternative to manage surgically recalcitrant CRS.

Acknowledgments

We thank Loren Matthews, Robb Muirhead, Paul Herde, Kevin Neuman, Dr Tim Kuchel and Carol Hewitt for their exceptional technical support at the Large Animal Research and Imaging Facility (LARIF). We also thank Lincoln Yardley from the Murray Bridge Abattoir for the supply of sheep heads for our optimisation study.

Funding: Sources came from the University of Adelaide, School of Medicine, Department of Otolaryngology Head and Neck Surgery, Adelaide, South Australia, Australia.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/ajo-18-97>). AJP serves as an unpaid editorial board member of *Australian Journal of Otolaryngology* and Consultant for Medtronic, ENT technologies, Tissium. SM reports other from Chitogel, during the conduct of the study; other from Chitogel, outside the submitted work; in addition, SM has a patent Chitogel issued. PJW reports other from Chitogel, during the conduct of the study; other from Chitogel, outside the submitted work; in addition,

PJW has a patent Chitogel issued. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study protocol was approved by the Animal Ethics Committee of The University of Adelaide and the South Australian Health and Medical Research Institute (SAHMRI). All reports on animal experiments have been conducted in accordance with institutional and national guidelines for the care and use of laboratory animals.

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doi: 10.21037/ajo-18-97

Cite this article as: Ooi ML, Drilling AJ, James C, Moratti S, Vreugde S, Psaltis AJ, Wormald PJ. Efficacy of anti-biofilm gel, chitogel-mupirocin-budesonide in a sheep sinusitis model. *Aust J Otolaryngol* 2020;3:16.