Methylglyoxal-augmented manuka honey as a topical anti-"Staphylococcus aureus" biofilm agent: safety and efficacy in an in vivo model

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Background: Bacterial biofilms are thought to contribute to recalcitrance in chronic rhinosinusitis (CRS) patients. Manuka honey (MH) and its active component methylglyoxal (MGO) have demonstrated antibiofilm activity in vitro. This study evaluated the safety and efficacy of these agents in an in vivo model.

Methods: To assess safety, ovine frontal sinuses were flushed twice daily for 14 days. In each sheep, 1 sinus was flushed with a panel of MGO concentrations ranging from 0.5 to 7.2 mg/mL alone and flushed with a panel of with 16.5% wt/vol MH enriched with MGO at the same range of concentrations (0.5–7.2 mg/mL; designated MH/MGO). Contralateral sinuses were flushed with saline control. Tissue morphology was assessed histologically and with scanning electron microscopy. Efficacy was tested by developing "Staphylococcus aureus" biofilms in sheep sinuses. Twice-daily irrigation for 5 days was commenced with either saline, MGO (0.5–3.6 mg/mL) alone, or MH/MGO (with 0.5–3.6 mg/mL MGO). Biofilm biomass was compared between the groups (n = 4) using LIVE/DEAD BacLight staining and confocal scanning laser microscopy.

Results: The results of the safety assessment, for normal sinuses treated with MGO alone or with MH/MGO (≤1.8 mg/mL) showed normal pseudostratified epithelium and cilia structure; however, higher concentrations caused cilia denudation and squamous metaplasia. As for efficacy, when compared to saline flush, treatment with MH/MGO at 0.9 mg/mL (0.608 ± 0.110 vs. 0.316 ± 0.197 µm²/µm², respectively; p = 0.015) and 1.8 mg/mL (0.676 ± 0.079 vs. 0.114 ± 0.033 µm²/µm², respectively; p = 0.001) significantly reduced biofilm biomass.

Conclusion: Sinus irrigation with MH/MGO at MGO concentrations between 0.9 and 1.8 mg/mL is both safe to mucosa and efficacious against S. aureus biofilm. MH/MGO irrigation could represent a viable treatment option for recalcitrant CRS. © 2014 ARS-AAOA, LLC.

Key Words: chronic rhinosinusitis; "Staphylococcus aureus"; biofilm; manuka honey; methylglyoxal; MGO

Manuka (Leptospermum scoparium) honey (MH) has shown strong antibiofilm activity against S. aureus in an in vitro setting. This effect has been shown to be primarily due to uniquely high levels of the phenol compound, methylglyoxal (MGO), within MH. Jervis-Bardy et al. demonstrated a synergistic antibiofilm effect when augmenting MH with additional MGO, requiring 16.5% wt/vol MH with 0.53 mg/mL MGO to achieve complete S. aureus biofilm eradication. Further studies have shown that the MGO concentration required for anti-S. aureus biofilm activity ranged between 0.5 and 3.6 mg/mL. Currently there are no studies that investigate the antibiofilm effects of MH and MGO within an in vivo model where interactions between MGO and biological tissue may alter its activity.

Human application of these agents is also restricted by concerns over the potential toxicity of MGO on living tissue. Current literature describes toxicity in some studies, whereas others report no deleterious effects. Furthermore, only 1 study has examined the effects of MH when applied to sinonasal mucosa, in which a 33% (wt/vol) MH solution of unspecified MGO content showed no epithelial injury. The effect that MGO may have on sinonasal mucosa was not examined in this, or subsequent studies.

The aim of this study is thus to evaluate the histological effects of MH and MGO on healthy sinus mucosa while also measuring its anti-S. aureus biofilm activity in a previously validated ovine model of biofilm-associated sinusitis.

Subjects and methods

Materials and animals

Safety and efficacy of MH and MGO were tested using an ovine model of biofilm-induced sinusitis with a standardized protocol developed at The Queen Elizabeth Hospital (TQEH). All procedures and protocols were approved by the Animal Ethics Committees of both the University of Adelaide and the Institute of Medical and Veterinary Science, South Australia.

Animals

Forty male merino sheep were used in this study, each assigned to a single arm of the study. Animals were of tooth age 2 to 4 (1–2 years old) and drenched to eradicate the parasite Oestrus ovis.

Honey and MGO

MH (590 mg/kg MGO concentration) was supplied by Watson and Son (Masterton, New Zealand) and was independently analyzed for MGO content by Hill Laboratories (Hamilton, New Zealand). MGO (40% wt/vol) was purchased from Sigma (Sigma Aldrich, St. Louis, MO). To make stock MH solution, MH was immersed in 0.9% saline to make a 16.5% wt/vol MH solution (total MGO content in solution = 0.1 mg/mL). This solution was augmented with additional MGO to make up the desired strengths for each treatment group (designated MH/MGO).

Bacterial inoculum

American Type Culture Collection (ATCC) 25923, a known biofilm-forming reference strain of S. aureus, was supplied by the Department of Microbiology, TQEH. A frozen glycerol stock was defrosted in 2 mL cerebrospinal fluid (CSF) broth (Oxoid, Adelaide, Australia) overnight before transfer to Columbian horse blood agar plates (Oxoid, Adelaide, Australia). These plates were incubated at 37°C for 24 hours, at which point a single colony forming unit (CFU) was subcultured on a second blood agar plate. Colonies from this subcultured plate were immersed into CSF broth and adjusted to 0.5 McFarlane units above baseline. This inoculum was then transported on ice for instillation into sheep frontal sinuses.

Safety arm

To test the effects of MH and MGO on healthy sinuses, frontal sinuses of 24 sheep were used.

Anesthetic protocol

General anesthesia (GA) was given by an experienced animal handler for all surgical procedures. Intravenous pethobartone (19 mg/kg) was administered as the induction agent. Sheep were then intubated and placed onto 1.5% to 2% inhalation isoflurane to maintain anesthesia.

Frontal trephination

The forehead was shorn and subsequently sterilized with Betadine (Mundipharma Pharmaceuticals BV, Amstelveen, The Netherlands). Mini-trephines (Medtronic, MN) were inserted bilaterally, 1 cm lateral to the midline, along a line crossing the midpoints of each bony orbit. Trephines were flushed with fluorescein (0.1 mL in 100 mL normal saline) to verify successful frontal sinus access and were subsequently capped.

Study groups and protocol

Sheep received twice-daily treatment flushes through the trephines for a period of 14 days. The right sinus of each sheep was irrigated with 50 mL 0.9% saline and served as a negative control. Within each group (n = 3) left sinuses were flushed with 1 of 5 MH/MGO combinations (50 mL of 0.5, 0.9, 1.8, 3.6, and 7.2 mg MH/MGO solution), shown described in Figure 1. MGO concentrations were selected based upon a previous in vitro efficacy range (0.5–3.6 mg/mL) plus a value representing double potency (7.2 mg/mL) to assess a broad safety range. Three additional groups received MGO-only solutions at 0.5, 3.6, and 7.2 mg/mL, diluted in 0.9% saline.
MGO-enriched MH against S. aureus biofilms

FIGURE 1. Safety study groups. Flow diagram describing the safety arm of this study. Within each sheep, control 0.9% saline was flushed through the right sinus. The left sinuses were flushed with experimental treatments (16.5% wt/vol MH enriched with MGO at 0.5, 0.9, 1.8, 3.6, or 7.2 mg/mL, respectively; and MGO alone at 0.5, 3.6, or 7.2 mg/mL, respectively), as shown. MGO = methylglyoxal; MH = manuka honey.

Acquisition of frontal sinuses
Animals were euthanized using sodium pentobarbital (>100 mg/kg), 24 hours after final sinus irrigation. The forehead skin and anterior table were elevated and each sinus was dissected away from the periosteal wall, excised, and placed directly into Dulbecco’s Modified Eagle Media (DMEM; Sigma Aldrich, NSW, Australia) for transport. Sinuses were then processed as required for analysis.

Safety analysis
For the safety study, primary outcomes were macroscopic assessment of collected sinuses in addition to histopathological analysis and evaluation of ciliary and tight junction morphology. General well-being of the sheep (appetite, behavior, weight change) represented the secondary measure.

For histopathological analysis, 10-mm × 10-mm sections were dissected and placed into 2% formalin solution. Samples were then mounted onto slides and stained with hematoxylin & eosin by an independent pathology group (Adelaide Pathology and Partners, Adelaide, Australia). Examination was conducted using a Nikon Eclipse 90i light microscope at ×40.0 magnification (Nikon Instruments Inc., Melville, NY).

For scanning electron microscope (SEM) studies, samples were cut into 5-mm × 5-mm pieces, sonicated in saline, then placed into SEM fixative (4% paraformaldehyde/1.25% glutaraldehyde in phosphate-buffered solution [PBS] with 4% sucrose) for at least 24 hours. The samples were subsequently washed 3 times in PBS and postfixed with 2% osmium tetroxide (OsO₄) for 1 hour. Tissue was dehydrated through a graded series of 70% to 100% ethanol. Samples were dried using hexamethyldisilazane (HMDS), mounted onto SEM stubs and sputter-coated with carbon gold. Analysis was conducted using an XL30 Field Emission Gun Scanning Electron Microscope (Philips, Eindhoven, Netherlands).

Efficacy arm
Anti–S. aureus biofilm activity of MH/MGO was tested on 16 sheep sinuses

Protocol
Endoscopic access to the frontal sinuses was achieved using a procedure as described. In brief, animals were placed under a GA and middle turbinates removed to reveal the anterior ethmoid complex, which was excised to expose the frontal sinus ostium. Once adequate hemostasis was achieved, animals were recovered and allowed a period of convalescence for minimum of 3 weeks. Animals were then given bilateral frontal trephinations and frontal sinus ostia were blocked endoscopically using Vaseline-soaked gauze (Kendall, Mansfield, MA). An inoculum of S. aureus was instilled into each sinus via the trephine, and biofilm was allowed to grow over the following 8 days. On day 8, the ostia were unblocked and treatment begun as per the study groups.

Study groups
Sheep were given twice-daily irrigation through trephines for a period of 5 days. Right sinuses received a control flush of 50 mL 0.9% saline. Within groups (n = 4) left sinuses received 50 mL MH/MGO treatment flushes of 4 different total MGO concentrations: 0.5, 0.9, 1.8, and 3.6 mg/mL (Fig. 2).
FIGURE 2. Efficacy protocol. Flow diagram of the efficacy arm protocol, outlining methodology and treatment groups. Within groups, the left sinuses were flushed with experimental treatments (16.5% wt/vol MH enriched with MGO at 0.5, 0.9, 1.8, or 3.6 mg/mL, respectively), as shown. The right sinuses of each sheep acted as an internal control, and were flushed at the same time with 0.9% saline. MGO = methylglyoxal; MH = manuka honey.

Tissue collection and sample preparation
Animals were euthanized as in the safety arm and sinus tissue was collected 24 hours after final sinus flush and dissected into 10 × 10 mm pieces. Each piece was stained with BacLight LIVE/DEAD Bacterial Viability kit (Invitrogen/Molecular Probes, Eugene, OR). Briefly, tissue was washed twice in Milli-Q (MQ) (Millipore, Darmstadt, Germany) water. Samples were then immersed in solution made up of 1.5 µL component A (Syto 9) and 1.5 µL component B (propidium iodide) in 1 mL sterile MQ water. Samples remained immersed in solution for 15 to 20 minutes at room temperature in complete darkness. After incubation, each tissue was rinsed twice in MQ water to wash out excess stain and was mounted on slides.

Biofilm imaging
Images of tissue samples were taken using a TCS SP5 confocal scanning laser microscope (CSLM; Leica Microsystems, Wetzlar, Germany) configured to a 488-nm argon laser and ×20 magnification water immersion lens. Two image-stacks (Z-stacks) of 2 individual pieces of tissue from each sinus were taken to make a total of 4 Z-stacks per sinus. The position of imaging corresponded to the areas of highest biofilm growth. Each Z-stack consisted of 110 individual images of the same area of tissue. The dimensions of each stack were 775.00 µm × 775.00 µm × 77.76 µm (width × length × height). The COMSTAT2 software (Lyngby, Denmark) was used to quantify biofilm volume in each Z-stack, as described.\textsuperscript{23,24}

Statistical analysis
Previous studies have defined a significant drop in biofilm biomass to be 60% reduction with treatment.\textsuperscript{10} Three sheep were required per arm to obtain a power of 80% with a significance level at \( \alpha = 0.05 \). Four animals per arm have been used to account for unforeseen early euthanasia. A paired \( t \) test was used to compare biomass between control and treated sinuses of sheep. Comparison between treatment groups was conducted using 1-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison posttest. Where applicable, numerical data is given in the form of mean ± standard deviation. All statistical analysis was performed using GraphPad Prism 5.0 software (San Diego, CA).

Results
Safety
All sheep remained healthy throughout the duration of the study with no weight change, appetite loss, or behavioral changes.

Gross features of sinuses
When exposed to MH solution with 7.2 mg/mL MGO or 7.2 mg/mL MGO alone, sinuses appeared grossly inflamed, with marked mucosal thickening and edema. These sinuses were filled with a thick, yellow-brown mucous that was negative for both bacteria and fungi. Sinuses treated with all other concentrations within the safety study appeared grossly normal when compared to control saline flush.

Histopathological analysis
Normal respiratory epithelium (pseudostratified columnar) was evident in sinuses flushed with control saline or with MH/MGO combination up to 3.6 mg/mL MGO (Fig. 3A-C). When treated with an MH/MGO concentration of 7.2...
mg/mL MGO (Fig. 3D), normal respiratory architecture was completely replaced by dense, stratified, squamous epithelial cells.

**SEM**

Figure 4A shows normal ciliary structure from a control (saline only) sinus. Ciliary ultrastructure appeared normal in sinuses receiving MH/MGO with up to 1.8 mg/mL MGO (Fig. 4B). Conversely, patchy ciliary denudation was observed in all specimens exposed to MH/MGO with MGO levels at or above 3.6 mg/mL (Fig. 4C). At MH/MGO concentrations of 7.2 mg/mL, impaired tight junctions with cellular detachment were also seen (Fig. 4D).

**MH/MGO combination vs MGO alone**

Sinuses treated with MH/MGO appeared identical to treatment with MGO alone at 0.5 mg/mL and 7.2 mg/mL MGO. At 3.6 mg/mL MGO, however, sinuses treated with MGO alone showed patchy stratification of the superficial mucosa in all sinuses (Fig. 5B). This was not seen with combination MH/MGO treatment at this concentration (Fig. 5A).

The safety results are summarized in Table 1.

**Efficacy**

When compared to saline control flushes, MH/MGO combination treatment at MGO concentrations of 1.8 mg/mL (0.676 ± 0.079 µm²/µm² vs 0.114 ± 0.033 µm²/µm², p = 0.001; paired t-test; Fig. 6C) and 3.6 mg/mL (0.608 ± 0.101 µm²/µm² vs 0.141 ± 0.039 µm²/µm², p = 0.001; paired t-test; Fig. 6D) significantly lowered biofilm biomass. Combination treatment with 0.9 mg/mL MGO also significantly reduced biofilm (0.608 ± 0.110 µm²/µm² vs 0.316 ± 0.197 µm²/µm², p = 0.015; paired t-test; Fig. 6B); however, the mean reduction in biomass was less than for higher concentrations. Treatment with the MH/MGO combination at 0.5 mg/mL MGO concentration resulted in no significant difference in biofilm biomass (Fig. 6A).

**Comparison between treatments**

There was no difference in biofilm biomass between flushing with 1.8 mg/mL MGO or 3.6 mg/mL MGO (p > 0.05; Bonferroni’s multiple comparison test; Fig. 7). Similarly, there was no statistically significant difference between a MGO concentration of 1.8 mg/mL and 0.9 mg/mL (p > 0.05; Bonferroni’s multiple comparison test).
FIGURE 4. Ultrastructure of sinus mucosa using SEM. Representative images from SEM of sinuses collected from animals within the safety arm. (A) Normal ciliary structure from a saline control sinus. (B) Normal ciliary appearance from a representative sinus receiving MH/MGO treatment (16.5% wt/vol MH enriched with MGO); this appearance was found at MGO concentrations of 0.5, 0.9, and 1.8 mg/mL. (C) Ciliary denudation; all cilia present are shortened to varying extents. The circled area represents the region of most stark shortening. This type of denudation was interspersed between areas of normal cilia in sinuses treated with MGO concentrations of 3.6 mg/mL and 7.2 mg/mL (both with MGO alone and with MH/MGO). (D) A lower magnification image showing an extended view of mucosa with completely effaced cilia. Note that cellular detachment is present with some cells budding out from the mucosa (white arrows). This was only seen in the 7.2-mg/mL MGO concentration. MGO = methylglyoxal; MH = manuka honey; SEM = scanning electron microscopy.

FIGURE 5. MH/MGO (3.6 mg/mL) combination treatment vs MGO alone (3.6 mg/mL). (A) Sinuses treated with MH/MGO (3.6 mg/mL) were marked by a loss of the fine, hair-like cilia. All other features of the mucosa are consistent with normal respiratory epithelium. (B) When exposed to MGO alone (3.6 mg/mL), however, epithelium displayed evidence of early transformation with stratification of the superficial layers (shown in square bracket). This represented the only concentration of MGO at which the combination treatment (MH/MGO) was different when compared to MGO alone. MGO = methylglyoxal; MH = manuka honey.

Table 2 summarizes the sinonasal safety and antibiofilm data collected from all concentrations of MH/MGO combination flushes.

Discussion

This study aimed to investigate the efficacy of MH and its active component MGO as a topical agent for the eradication of *S. aureus* biofilms in the setting of in vivo sinusitis while also assessing safety of applying high levels of MGO. Our results demonstrate a positive correlation between MGO and both antibiofilm activity and mucosal damage. There was also evidence that MGO’s toxic effects are partially reduced when exposed concurrently with MH compared to MGO alone.
### TABLE 1. Summary of effects of treatment on healthy sheep sinuses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>General sheep Condition</th>
<th>Sinus gross features</th>
<th>Sinus histopathology</th>
<th>Ciliary morphology (SEM)</th>
<th>Tight junction morphology (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Unchanged</td>
<td>Normal</td>
<td>Normal respiratory mucosa</td>
<td>Normal</td>
<td>Intact</td>
</tr>
<tr>
<td>MH/MGO (0.5 mg/mL)</td>
<td>Unchanged</td>
<td>Normal</td>
<td>Normal respiratory mucosa</td>
<td>Normal</td>
<td>Intact</td>
</tr>
<tr>
<td>MGO only (0.5 mg/mL)</td>
<td>Unchanged</td>
<td>Normal</td>
<td>Normal respiratory mucosa</td>
<td>Normal</td>
<td>Intact</td>
</tr>
<tr>
<td>MH/MGO (0.9 mg/mL)</td>
<td>Unchanged</td>
<td>Normal</td>
<td>Normal respiratory mucosa</td>
<td>Normal</td>
<td>Intact</td>
</tr>
<tr>
<td>MH/MGO (1.8 mg/mL)</td>
<td>Unchanged</td>
<td>Normal</td>
<td>Normal respiratory mucosa</td>
<td>Normal</td>
<td>Intact</td>
</tr>
<tr>
<td>MH/MGO (3.6 mg/mL)</td>
<td>Unchanged</td>
<td>Normal</td>
<td>Normal respiratory mucosa</td>
<td>Patchy ciliary denudation</td>
<td>Intact</td>
</tr>
<tr>
<td>MGO only (3.6 mg/mL)</td>
<td>Unchanged</td>
<td>Normal</td>
<td>Stratification of epithelial lining</td>
<td>Patchy ciliary denudation</td>
<td>Intact</td>
</tr>
<tr>
<td>MH/MGO (7.2 mg/mL)</td>
<td>Unchanged</td>
<td>Inflamed</td>
<td>Squamous metaplasia</td>
<td>Patchy ciliary denudation; cellular detachment</td>
<td>Intact</td>
</tr>
<tr>
<td>MGO only (7.2 mg/mL)</td>
<td>Unchanged</td>
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</tr>
</tbody>
</table>

MGO = methylglyoxal; MH = manuka honey; SEM = scanning electron microscope.

The present study was, to our knowledge, the first to examine the dose-dependent effects of MGO on the sinonasal mucosa of a live animal model. Although previous studies had investigated MH application to this region, the MGO content of the honey was unmeasured. This creates difficulty in interpretation of such results because MGO levels among MHs can vary considerably. Similar to previous in vivo studies, tissue damage increased proportionally to MGO concentrations, reflecting its toxic nature. A wealth of in vitro literature currently describes potential

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**FIGURE 6.** Staphylococcus aureus biofilm biomass scatter plots. Comparison of means between control saline and MH/MGO at MGO concentrations of (A) 0.5 mg/mL, (B) 0.9 mg/mL, (C) 1.8 mg/mL, and (D) 3.6 mg/mL. Biofilm biomass is plotted on the y-axis, comparing control flush (in blue) with treatment (in red). Individual points on graph indicate average biomass within a single sinus. Bars represent mean with standard deviation. MGO = methylglyoxal; MH = manuka honey.
mechanisms of MGO toxicity, including free radical production, enzyme dysfunction, oxidative stress, and DNA damage.16 The effects of translation to in vivo models are less clear, with several studies reporting differing outcomes. One such study applying MGO to rat peritoneum found dense adhesion formation and animal death at 20 mmol/L (1.44 mg/mL) levels of MGO.17 Interestingly, we report no changes to sinus mucosa at similar levels and only microscopic changes at more than double this concentration. Possible explanations for this may lie in the ability of the sinuses to clear substances via the mucociliary escalator, or their relative resilience against foreign molecules compared to the peritoneum. Irrespective of cause, this suggests that different tissues respond varyingly to MGO and that higher levels of MGO than previously considered may be delivered to sinuses without negative effect. Tissue damage continues to remain a concern at the highest concentrations, however, because squamous metaplasia most often represents an attempt to protect the mucosa and underlying tissue from noxious stimuli and indicates a reactive response to toxic levels of MGO.26 We also report partial reduction of MGO’s adverse effects when administered concurrently with MH compared to MGO alone, suggesting that an MH/MGO combination therapy is likely to represent the most viable clinical application. Previous studies indicate that MH possesses anti-inflammatory and free radical scavenging properties which may help to explain the observed attenuating effects on MGO.27,28 The exact extent and mechanism of this potential protective effect have not been explored and remains an area for future research.

The results of this study confirm the anti–S. aureus biofilm properties of MGO documented in vitro,4 with a minimum “biofilm-cidal” concentration of 0.9 mg/mL in a combination treatment. Although existing literature has reported the use of MH as an antibiotic in human CRS patients, little evidence has previously existed to support its function as an antibiofilm agent in vivo. Our study has shown that antibiofilm activity with MH/MGO can be achieved in vivo if 16.5% MH is augmented with additional MGO. Within the context of CRS, unlike superficial wounds and burns, neat honey is impractical as it may not adequately reach target areas within sinuses, and residually block the nasal airway. The enriching of MH with an additional active ingredient allows for application of greater MGO without increasing the honey concentration. Because honey can also be intolerable to sinonasal mucosa,4 augmentation represents a novel approach for delivery of biofilm-cidal levels of MGO in an MH solution. Studies by Jenkins et al.29,30 have found that MH/MGO inhibits cell division and reduces expression of stress protein A in planktonic S. aureus, suggesting a possible mechanism for antibacterial action. What additional effects they may have on biofilms, in which there exists a subset of nondividing dormant bacteria, is yet to be determined. Although our study focused on a single species, both MH and MGO display a broad spectrum of antibacterial activity including S. aureus and P. aeruginosa,31 the 2 most commonly cultured organisms in severe, recalcitrant CRS. Given the often polymicrobial nature of sinus disease, benefits are readily apparent with agents such as MH/MGO that may combat several pathogenic organisms simultaneously.

Biofilm-associated CRS continues to pose significant challenges in the field of rhinology. Despite advances in both medical and surgical therapies, eradication of these bacterial communities has not been achieved3,31 due to their inherent resistance to traditional topical and oral antibiotics.32 MH is a potential agent that can be used repeatedly without substantial risk for microbial resistance.33 We have found both antibiofilm activity and tissue toxicity to directly increase with MGO concentration. Optimal clinical application should therefore titrate MGO carefully. Our
results suggest that MH/MGO with MGO concentrations between 0.9 and 1.8 mg/mL would provide significant antibiofilm activity without damaging sinonasal mucosa. Furthermore, patients have previously shown good tolerance to MH/MGO when applied to the sinonasal mucosa. Moreover, patients have previously shown good tolerance to MH/MGO when applied to the sinonasal mucosa. Finally, all statistical analysis was performed with the aid of Dr. Jessica Kasza, Department of Biostatistics, University of Adelaide, Adelaide, South Australia.

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