Research Paper

The association of Leptospermum honey with cytokine expression in the sinonasal epithelium of chronic rhinosinusitis patients

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Abstract *Objective:* To identify the differences in cytokine expression between sinonasal tissue from patients treated with Leptospermum (Manuka) honey (LH) irrigation versus normal saline irrigation twice-daily for twelve weeks following sinus surgery (FESS).
Methods: Forty-six CRS patients were recruited. Sinus tissue biopsies were collected during FESS and then at 5 and 12 weeks postoperatively during the course of treatment. A multiplex cytokine assay quantified the abundance of 17 cytokines in biopsied tissue. Cytokine expression fold-change was analyzed between each time point using a robust linear regression model and compared between the two treatment groups.
Results: Compared to the saline irrigation group, five cytokines were differently expressed ($CI = 95\%$) in sinonasal tissue obtained from subjects in the LH irrigation group during the 12-week treatment period. Cytokines IL-6 ($P = 0.0400$), IL-8 ($P = 0.0398$), MCP-1 ($P = 0.0284$), and MIP-1β ($P = 0.016$) were significantly increased in the LH irrigation group

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compared to the saline irrigation group. IL-13 was significantly increased in the saline irrigation group compared to the LH group ($P = 0.0086$).

Conclusion: LH may potentially act to modulate the expression of IL-6, IL-8, IL-13, MCP-1 and MIP-1β in sinonasal tissue.

Introduction

Leptospermum (Manuka) honey (LH) is a natural immuno-modulatory and antimicrobial agent. It has been well documented as a therapy for burns and surgical wounds.[1] However, it has only recently been introduced as a potential treatment option for chronic rhinosinusitis (CRS).[2] CRS is an inflammatory disorder of the nose and paranasal sinuses that affects nearly thirty million people in the US.[3] The source of inflammation is thought to be a product of problems both innate and environmental.[4] CRS patients may require years of intermittent courses of antibiotics and steroids for symptom management.

Functional endoscopic sinus surgery (FESS) has been shown to improve symptoms and quality of life in those who fail medical management.[5] Meticulous postoperative follow-up and treatment of CRS patients is critical to attaining positive surgical outcomes. There is good evidence that FESS results in the eventual repair of diseased mucosa.[5–7] There are two components of postoperative care that must be taken into account: surgical wound repair and the underlying chronic inflammatory process. Very little is known regarding surgical wound repair following sinus surgery. Mucosal healing is typically a well-organized four-step process involving inflammation, cell proliferation, matrix deposition and remodeling.[8] Following surgical debridement, the coagulation phase activates an inflammatory process beginning with neutrophils in the first forty-eight hours, followed by an influx of macrophages between days three and five.[8] Macrophages are primarily responsible for cellular debridement but also stimulate proliferation of fibroblasts and angiogenesis.[9] The sinonasal epithelium then undergoes a process of tissue remodeling involving a number of proteinases that degrade the extracellular matrix and subsequently cause the wound to contain a higher proportion of type I collagen.[10] The wound repair process following surgery takes at least six months before mucosal integrity is restored.[5]

There has been considerable effort made to understand the inflammatory process that underlies different subtypes of CRS.[11] This interest is due, in part, to a desire to target post-operative pharmacotherapy. Different inflammatory triggers have been hypothesized. These include the immune barrier hypothesis, the superantigen hypothesis and the fungal hypothesis.[12] The presence of these triggers may persist in the post-operative period. Consequently, post-operative treatment that both promotes wound healing and addresses the underlying chronic inflammatory disorder is desired.

Our institution performed the first *in vivo* assessment of LH's clinical use in sinonasal care.[13] This clinical study showed symptomatic improvement in patients taking LH versus those using saline solution, but no difference in endoscopic scores between the two groups was noted. It was therefore unclear whether honey was having an impact on the inflammation in the underlying mucosa or if the symptomatic improvement was a placebo effect. Given the anti-inflammatory and antimicrobial properties of LH, the objective of this study was to determine if LH had an immunomodulatory effect on the sinonasal mucosa of post-operative CRS patients.

Materials and methods

Subjects

Patients undergoing treatment at a tertiary rhinology centre in Canada were invited to participate in this study. Patients were included if they had been diagnosed with CRS (with or without polyps) based on the Canadian guidelines criteria and were undergoing FESS after failing at least three months of medical management.[14] Complete FESS was defined as a patient requiring a frontal sinusotomy (Draf Type IIA), ethmoidectomy, maxillary antrostomy, and sphenoidotomy. Patients were excluded from this study if they had comorbid diabetes, cystic fibrosis, immunodeficiency, were undergoing treatment for a sinonasal tumor, or were currently being treated with anticoagulants, anti-hypertensives, oral and/or topical corticosteroids. Patients were also excluded if they had a known allergy to honey or bee stings. Baseline demographic and clinical variables such as age, sex, Lund–Mackay CT score, concomitant medications and comorbidities were obtained from health records. This research was conducted under the auspices of the Providence Health Care Research Ethics Board (PHCREB) and Health Canada.

Pre-operative washout period and randomization

All patients enrolled in the study were instructed to refrain from taking oral or topic nasal steroids or antibiotics twenty-eight days prior to surgery. Patients were excluded from the study if these medications were taken within twenty-eight days of their surgery. One the day of surgery, a closed envelope system was used to randomize patients to LH versus saline nasal irrigation. Patients deemed to requiring additional medical management (i.e. intranasal

corticosteroids) upon postoperative assessment were removed from the study given the confounding effect it would have on the cytokine tissue response observed in tissue samples obtained.

LH irrigation

LH (5%−7% concentration) used for this study was obtained from Honey Doc® (Honey Doc Products Inc., Vancouver, Canada). Patients irrigated their sinonasal cavities by placing the solution into a 240 ml *power rinse*® bottle nasal irrigator, purchased from Honey Doc®. Patients were instructed to start irrigating their sinuses twice a day for three months beginning as soon they were discharged from the hospital.

Normal saline irrigation

Distilled water reconstituted with pH-balanced sodium chloride and sodium bicarbonate mixture (made by dissolving the contents of one Sinus Rinse™ (NeilMed Pharmaceuticals Inc., Markham, Canada)) sachet into a 240 ml *power rinse bottle* nasal irrigator, purchased from Honey Doc®. Patients were instructed to start irrigating their sinuses twice a day for three months as soon they were discharged from the hospital.

Sample collection

Patients were biopsied intraoperatively and again at the five and twelve-week post-operative clinic visits. Biopsies collected in the clinic were performed under topical anesthesia (4% xylocaine and 0.05% oxymetazoline, two sprays into the appropriate side of the nose). Tissue was collected in cryogenic ampules (Fisher Scientific) and immediately stored in liquid nitrogen. Sinus tissue samples were collected under endoscopic guidance using a pediatric 2.3 mm nasal endoscope and 45-degree pediatric biopsy forceps. Samples were consistently obtained from the anterior ethmoid sinus cavity. This site was chosen because of its ease of accessibility postoperatively.

Tissue analysis

Prior to analysis, biopsied tissue was homogenized in a protein extraction reagent used to create cell lysates (Tissue Extraction Reagent, Invitrogen). Tissue samples were analyzed for cytokine concentration with a Luminex system using a Bio-Plex Pro Human Cytokine Assay (Bio-Rad Laboratories) platform in accordance with the manufacturer's instructions. The 17-plex assay kit detects key inflammatory cytokines, including some known to be involved in CRS-related inflammation. The specific human cytokine targets for this assay include: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1 (MCAF), MIP-1α, MIP-1β, TNF-α. All cytokine data was normalized to total protein levels using the BCA protein assay kit (Pierce, Thermo Scientific). Each assay was performed in duplicate at a 1:4 dilution of sample to buffer reagent.

Data analysis

Cytokines with little variation across all subjects and time points are not likely to be differentially expressed and therefore half of the least variable cytokines were removed prior to downstream statistical analysis. Three independent comparisons: Baseline *vs* 5-weeks, 5-weeks *vs* 12-weeks and Baseline *vs* 12-weeks were performed using robust linear regression in the Linear Models for Microarrays (limma) R package (version 3.10.3).[15] Robust regression reduces the effects of outliers on the regression coefficients estimated using least-squares.[16] A *P value* of 0.05 was used to determine statistically significant differences between cytokine fold-change levels at each time point.

Results

Data pre-processing

Forty-six patients were recruited to this study ($n = 46$). Five subjects withdrew from the study at the 5-week postoperative visit because they opted not to provide any tissue biopsies during the treatment period ($n = 5$). Of the remaining included cohort, a tissue biopsy was obtained from 41 subjects intraoperatively and at the 5-week postoperative visit ($n = 41$). Baseline demographic and clinical characteristics of the included cohort are summarized in Table 1. Five subjects were deemed to require topical nasal corticosteroid intervention at the 5-week postoperative visit based on endoscopic mucosal inflammation scores ($n = 5$). This group was comprised of three patients in the LH treatment arm and two patients in the saline treatment arm. These five subjects were commenced on nasal corticosteroid therapy after providing a biopsy at the 5-week visit and were excluded from providing an additional biopsy at 3 months postoperatively. Nine subjects opted out of providing a tissue biopsy at the 3-month postoperative visit ($n = 9$). The remaining twenty-seven subjects provided a biopsy up to 3-months postoperatively ($n = 27$).

The standard deviation of each cytokine across all samples was computed and fifty percent of the most variable cytokines were identified. Cytokines with minimal variation across all subjects were not likely to be differentially expressed and therefore were removed. The group of excluded cytokines was comprised of: IL-2, IL-4, IL-7, IL-9, IL-10, IL-12, IL-15, IL-17, IFN-γ, MIP-1α, and TNF-α. The remaining nine (highly variable) cytokines that were retained for downstream analyses included: IL-6, IL-8, MCP-1, MIP-1β, IL-13, IL-1β, GM-CSF, IL-5, G-CSF.

Longitudinal effect of LH on cytokines relative to saline irrigation

Each pair of time points was considered in a separate linear model (Table 2). Five weeks after treatment, IL-6, IL-8 and MCP-1 significantly changed in the honey groups compared to the saline group (Fig. 1). Cytokines IL-8 and MCP-1 significantly increased from baseline values in biopsies obtained from the LH group relative to subjects in the saline group. IL-6 increased in the LH group and decreased in the

Table 1 Baseline and clinical characteristics of Leptospermum honey and normal saline treatment cohorts.

| Groups | Age[a] | Sex (%) | | Asthma (%) | Smoking habits (%) | | | Mean CT-score (Lund—Mackay system)[a] | Polyposis (%) |
		Male	Female		Current smoker	Ex-smoker	Non-smoker		
LH group ($n = 26$)	47.07 ± 13.69	13 (50)	13 (50)	5 (19)	4 (15)	4 (15)	18 (69)	13.98 ± 6.89	8 (31)
NS group ($n = 15$)	51.67 ± 13.23	8 (53)	7 (47)	3 (20)	1 (7)	2 (13)	12 (80)	15.36 ± 6.62	5 (33)

[a] Values are presented as mean ± standard deviation. LH: Leptospermum honey; NS: normal saline.

Table 2 Comparison of cytokine expression between Leptospermum honey and saline irrigation treatment groups.

| Cytokine | Fold-change (pg/ml) | | P value | | | P value | | | P value |
| | Baseline *vs* 5-weeks | | | Baseline *vs* 3-months | | | 5-weeks *vs* 3-months | | |
	Saline ($n = 15$)	LH ($n = 26$)		Saline ($n = 8$)	LH ($n = 19$)		Saline ($n = 8$)	LH ($n = 19$)	
MCP-1	0.00	1.14	0.0284[a]	0.14	−0.52	0.0700	0.17	0.57	0.531
IL-6	−0.17	0.04	0.0400[a]	0.11	0.02	0.0726	0.01	0.04	0.700
IL-8	0.04	0.74	0.0398[a]	0.10	−0.33	0.3076	−0.04	0.24	0.294
IL-13	−0.10	−0.02	0.1120	0.10	0.03	0.0086[a]	−0.01	0.00	0.597
IL-1β	0.01	0.14	0.2486	0.00	−0.01	0.2984	0.00	0.06	0.370
MIP-1β	−0.14	0.15	0.2992	0.40	0.09	0.3488	−0.39	0.55	0.016[a]
GM-CSF	−0.09	−0.10	0.5192	0.18	0.00	0.1157	−0.09	−0.09	0.263
IL-5	−0.01	0.00	0.8073	0.02	−0.01	0.0789	0.01	−0.02	0.532
G-CSF	0.11	0.05	0.9368	−0.02	−0.20	0.2295	0.12	−0.17	0.220

[a]$P < 0.05$.
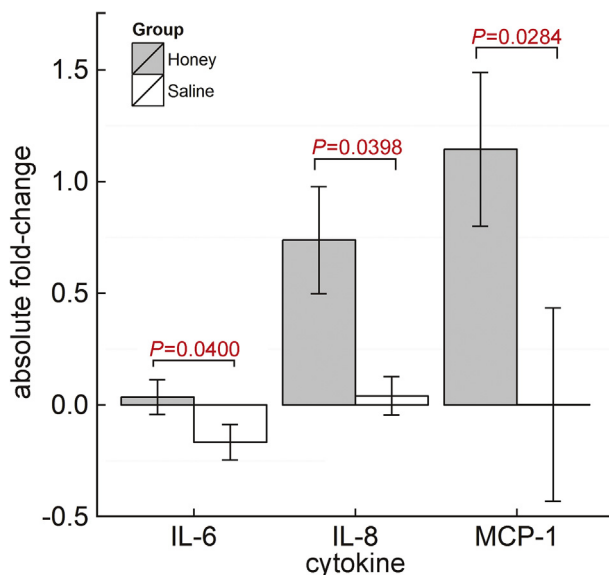
Fig. 1 Cytokine expression fold-change from baseline to the 5-week postoperative clinic visit in Leptospermum honey (LH) and Saline irrigation treatment groups.

saline group five weeks after treatment. The association of LH treatment with IL-6 and MCP-1 expression was somewhat preserved at twelve weeks of treatment, however, this association did not reach statistical significance ($P = 0.07$). MIP-1β was the only cytokine significantly different between the five weeks and twelve weeks clinic visit ($P = 0.016$). Between baseline and 12 weeks postoperatively, there was a statistically significant increase in IL-13 in the saline group relative to the LH group ($P = 0.008$).

Discussion

Overview of results

The cytokines IL-6, IL-8, and MCP-1 were significantly up regulated from baseline levels in the cohort receiving LH irrigation treatment compared to those in the saline irrigation group. Interestingly, IL-13 was significantly increased from baseline levels in the saline irrigation group compared to the LH group. Significantly different levels of IL-8 observed in the group receiving LH at five weeks may be attributed to the generation of oxidative species from hydrogen peroxide found in LH.[17] Reactive oxygen species (ROS) are known to activate the transcription factor NF-κB that is responsible for promoting macrophage activity and up-regulation of the pro-inflammatory cytokine, IL-8.[18,19] If kept unchecked, an over-amplification of the immune response by ROS can be harmful. However, it is thought that the antioxidant properties of LH are able to counteract the activity of ROS over time.[17] MCP-1 and MIP-1β are pro-inflammatory cytokines and have yet to be identified as cytokines associated with the effect of LH. Their up-regulation in the LH group at five weeks and twelve weeks, respectively, is open to interpretation. In previous investigations, MCP-1 was identified as a mediator of early-phase wound healing, and MIP-1β, a mediator of late-phase wound healing.[20] The shift in prominence of specific

cytokines: IL-6, IL-8, and MCP-1, down to lower levels of significance in the LH group at twelve weeks, can be explained by the phases of wound healing. During the early phase between surgery and the five-week postoperative period these cytokines serve a valuable purpose by promoting macrophage activity for wound debridement. They are also known to be responsible for the re-epithelialization and angiogenesis processes that are critical in the early phases of wound healing.[21]

Although it is counterintuitive to imagine that the up-regulation of both pro- and anti-inflammatory cytokines can be productive in a chronically inflamed environment, this is often the impetus needed to progress a recalcitrant wound towards cell proliferation and the healing phase.[22–24] This represents a key concept of LH's function and requires a paradigm shift in how CRS patients are treated following FESS. Global reduction of inflammation with systemic steroids during the early postoperative period may not be the best treatment modality for all CRS patients. The potential benefits of LH must be weighed in each case. It is unclear whether the benefits of LH are masked by the concomitant use of steroids and this would require further investigation.

LH in the context of post-FESS care

Meticulous postoperative care is important to the long-term success of functional endoscopic sinus surgery. Adequate treatment of mucosal inflammation after surgery by either medical means or with debridement has been found to nearly eliminate the need for revision surgery.[25] It is important to recognize that patients require close post-operative care during this period. In the early post-operative phase, clinicians must devise a treatment plan that targets the surgical wound as well as the underlying chronic inflammatory processes of CRS. There are few strict guidelines for optimal early postoperative care and this is may be attributed to heterogeneity of the patient population and the variability of outcomes following FESS.

General treatment options in the early post-operative phase include sinus cavity debridement, saline irrigation, systemic steroids, topical steroids, oral antibiotics, topical decongestants and drug-eluting spacers. A study by Rudmik et al[26] provided an evidence-based review and recommendations regarding the aforementioned options. The review showed that sinus cavity debridement, saline irrigation and topical nasal steroids were recommended.[26] Each of these recommended options play an integral role in the healing of the sinonasal mucosa following surgery and have varying effects on the four-step process of normal mucosal healing (inflammation, cell proliferation, matrix deposition and remodeling).[27] Sinus cavity debridement and saline irrigation allow for the removal of old blood clots, secretions and unabsorbed packing that can perpetuate harmful inflammation.[28] Topical nasal steroids have been shown to decrease cell proliferation by inhibiting fibroblasts, therefore, minimizing synechiae formation and preserving ostial patency.[29,30]

LH irrigation could have a role to play in the arsenal of postoperative treatment following FESS.[14] This study is a novel investigation into the use of LH for CRS, and the first of its kind to assess the effect of LH on paranasal sinus mucosa *in vivo*. The findings presented here are corroborated by several other investigations of the immunomodulatory and wound healing properties of LH.[29,30] LH has a long history as a medicinal agent and is a well-proven wound healing agent.[31–34] It has been suggested that the effectiveness of honey on wound healing may be secondary to the stimulation of key inflammatory cytokines.[35] Our study is advantageous in that it provides snapshots of these dynamic inflammatory mediators over short-term (five weeks) and long-term (twelve weeks) periods of exposure to LH. In a time-course study, monocytic cells exposed to LH over a 24-h period were observed to increase expression of IL-6, TNF-alpha and IL-1β compared to a control treatment.[35] IL-6 has previously been identified as a key mediator of the wound healing process and in the present study, it was found to be up-regulated in the LH irrigation treatment group (Fig. 1).[36–39] Tissue healing can be hampered by a diversity of factors and this is especially true in a chronically inflamed environment[40] Macrophages are critical to proper wound healing and their depletion can hamper wound debridement that is critical during the acute phase of wound healing.[21]

Topical nasal steroids are commonly prescribed to patients during the postoperative period to encourage wound healing and treat local inflammation.[26] However, patients are often skeptical of the effectiveness and wary of side effects. A large proportion of patients at our centre have expressed their dissatisfaction with steroid use and embrace the idea of a natural option. Elucidating the impact of any treatment on the immune response is vital to picking the appropriate drug for inflammatory diseases. In the case of CRS, the inflammatory cascade varies between the presence or absence nasal polyposis, antigen triggers, and even ethnicity.[41] Therefore, it is important to understand which cytokines within the inflammatory cascade are impacted by LH in order to help clinicians decide which patients may benefit most from LH treatment.

Limitations

Our study utilized a concentration of 5%–7% LH that was commercially available to us, therefore, it was clinically relevant to investigate the immunomodulatory effect of honey at this concentration. However, it is unknown whether the immunomodulatory effect would have been different at differing honey concentrations. The optimal concentration for sinonasal application has yet to be determined. The concern with honey at high concentrations is the hyperosmolarity effect, which can cause a burning or stinging sensation.[42] Jervis-Bardy et al[2] suggested a 16.50% concentration to balance the bactericidal activity and hyperosmolarity effect *in vitro*. However, other studies have shown that the minimum inhibitory concentration of LH can be achieved at 2% concentration.[43,44] The ideal concentration requires investigation in which it promotes bactericidal activity and positive immunomodulatory effects while minimizing the effect of hyperosmolarity. Tolerability of the biopsy became an issue at postoperative visits. A number of patients declined the biopsy because of discomfort or the notion of a biopsy. A

more equal distribution of biopsies samples between visits would strengthen the results of the twelve-week visit.

Conclusion

LH is a known immunomodulator and was associated with a cytokine response that may be beneficial to the wound healing process. Several cytokines identified in this study have been associated with the effect of LH in previous literature, however, others are novel cytokines that require further investigation. Since LH appears to target specific cytokines, and given the heterogeneity of CRS patients, this therapy may not yield the same benefits to all patients undergoing FESS.

Authorship contributions

JM contributed to the conception, design and coordination of the study, prepared cytokine assays, conducted data analysis, and contributed to manuscript preparation. AVT conceived the study, participated in the design and coordination, conducted data analysis, and contributed to manuscript preparation. VSS contributed to study design, patient recruitment, and manuscript preparation. AS carried out the statistical analysis of data and contributed to manuscript preparation. ST provided mentorship, helped with statistical analysis and contributed to manuscript preparation. CG provided mentorship, contributed to the design and coordination of the study, and contributed to manuscript preparation. ARJ provided mentorship, participated in the design and coordination of the study and contributed to manuscript preparation. All authors read and approved the manuscript.

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