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RESPIRATORY INFECTIONS (F ARNOLD, SECTION EDITOR)

The Effect of Spray Cryotherapy on Microbial Biofilms in Chronic Rhinosinusitis

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Abstract

Purpose of Review Microbial biofilms seem to play an active role in the pathogenesis of chronic rhinosinusitis (CRS). They represent an adaptive defense resource enabling resistance to antibiotics and host defense mechanisms. Biofilms are thought to be accountable for refractory cases of sinusitis by perpetuating local inflammation. The objective of this study was to assess the effectiveness of spray cryotherapy as a biofilm disruption agent in CRS in an in vitro model.

Recent Findings A total of 23 patients with CRS undergoing endoscopic sinus surgery (ESS) were included. Rhinosinusal mucosa samples were harvested. Half of sample was left intact while the other half was treated with spray cryotherapy, so patients served as their own witnesses. Subsequently, they were processed to hematoxylin-eosin (HE) and toluidine blue (TB) staining and analyzed by light microscopy. Biofilms were detected in 17 of 23 patients with CRS. Staining by HE showed strong correlation with the results of TB staining protocol. The in vitro CRS study demonstrated that spray cryotherapy removed polymicrobial biofilms from the mucosa surface in 70.6% of cases and induced important structural changes in the remaining samples.

Summary Thus far, cryotherapy has proven to be a reliable method for the disruption of microbial biofilms in CRS with nasal polyps, in vitro conditions. Spray cryotherapy could be a considerable benefit in the management of recalcitrant CRS.

Keywords Biofilm · Chronic rhinosinusitis · Hematoxylin-eosin staining · Cryotherapy

Introduction

Chronic rhinosinusitis (CRS) is one of the most common diseases of the upper respiratory tract, affecting approximately 12% of the adult European population [1••, 2••, 3, 4]. It is defined as the inflammation of the rhinosinusal lining lasting for more than 12 weeks [5] and comprises a heterogeneous group of conditions with the presence of important variation among the individuals, that make difficult the management of the disease [1••], with significant socio-economic impact. CRS has been associated with impaired quality of life, depression, anxiety, sleep disorders, sexual dysfunction, and high treatment costs [3, 6–9]. Moreover, the inability to effectively treat CRS can lead to severe endo-orbital or endo-cranial complications and even death. The pathology of this disease is conditioned by multiple factors, including dysfunction of local pathways for regulation and resolution of inflammation, microbial colonization (biofilms, osteitis), fungus, superantigens, allergy, and other abnormal adaptive immunological reactions, as well as metabolic abnormalities such as aspirin sensitivity [10•]. Recent evidence supports the theory that microbial biofilms play a critical role in exacerbating and perpetuating the inflammatory process of the sinusal mucosa [10•, 11, 12••, 13–18].

Bacterial and fungal colonies exist in two main forms: as free planktonic cells or as complex biofilms [19–21]. The latter are highly organized microbial structures characterized by extremely high resistance to antibiotics, host immune reactions, and various chemical and physical agents [12••, 13, 22]. Results based on laboratory culture, electronic microscopy, and confocal laser scanning were variously cited as evidence that biofilms are important in the pathogenesis of CRS, particularly in cases refractory to paramount therapy [23, 24].

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Biofilm eradication strategies are increasingly important due to their high antimicrobial resistance and the concomitant slowdown in the development of new antibiotics [22, 25•].

Biofilm formation is a crucial virulence factor for a wide variety of microorganisms that represents source chronic infections [26••]. The biofilm development and drug tolerance requires considerable obstacles for the use of conventional antimicrobials and denotes the necessity for developing alterative therapeutic strategies with and increased efficacy, including surface modification, physical disruption, or new nanoparticle-based systems [26••].

Spray cryotherapy using low-pressure liquid nitrogen is a new technique used in the management of premalignant and malignant esophageal lesions that has proven to be also effective and safe when applied in the paranasal sinuses [27]. Considering the above, low-dose spray cryotherapy could be an effective and accessible treatment in CRS through the destructive process it could induce on the rhinosinusal biofilms.

This study aims to investigate the impact of spray cryotherapy, an innovative and non-invasive method to counteract the resistance to antibiotic caused by bacterial biofilm formation in CRS, to evaluate the outcomes of patients using this approach.

Material and Method

Studied Population

We performed a prospective blinded comparative study, conducted in the Department of Otorhinolaryngology of the CF Cluj-Napoca Clinic (affiliated to the University of Medicine and Pharmacy "Iuliu Haţieganu" Cluj-Napoca). The study group included 23 patients with bilateral symmetric chronic sinusitis, with or without nasal polyps, who did not respond to the optimally applied drug treatment, requiring endoscopic sinus surgery (ESS).

The group was comprised of adults with CRS, diagnosed based on disease history, physical examination, endoscopic examination, and paraclinical investigations, in accordance with the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 (EPOS2012) [5]. Patients under 18 years of age, patients known with acute upper respiratory tract infections, immunodeficiency or predisposing conditions (ciliary dyskinesia, cystic fibrosis), and patients who underwent a course of antibiotics or systemic corticosteroids 4 weeks prior to sample collection were excluded from the study. Preoperative corticoid or nonsteroidal intranasal decongestants were permitted.

Patients were divided, based on clinical aspect, into cases of chronic rhinosinusitis with nasal polyps and without nasal polyps. Medical data regarding allergic status, bronchial asthma, aspirin intolerance, previous ESS, topical steroid treatment, and systemic antimicrobial therapy was documented before surgery. Extension of disease was assessed by computerized tomography (CT) and classified according to the Lund-Mackay severity score. All patients gave an informed consent after explaining the nature of the experimental procedures, and the study was approved by the local ethics committee.

Sampling

Prior to surgery, bacteriological swabs were first obtained using the procedure described by Nadel et al. [28]. A topical anesthetic agent was applied in the nasal cavity, after this, under direct endoscopic guidance, a thin, flexible, calcium alginate swab was inserted and led to the site most profuse in purulent secretions or the medial nasal meatus if these were not visualized. Great care was taken to avoid contact with the lateral and septal nasal walls or the nasal vestibule to avoid contamination. The swab was immediately placed and carried into a Standard Calcium Alginate Tip Dry Transport System. Samples were plated for qualitative growth on culture media: Colombia agar with 5% ram blood, agar chocolate, and Mac Conkey agar. They were incubated under standard conditions, at 37 °C in 5% CO_2 atmosphere, for 24–48 h and tested for sensitivity to various antibiotics.

As a next step, during the endoscopic sinus surgery, from each patient, we harvested fragments of inflamed respiratory mucosa (with or without polypoid transformation) from the middle meatus and the ethmoidal sinus. To reduce the risk of bias, the interventions were performed by the same Ears-Nose-Throat surgeon (ENT surgeon) and the operation were meticulously performed to minimize bleeding and to eliminate the need for multiple irrigations during surgery. The removal was gently performed with strait or curved Blakesly nasal forceps to prevent surface injury or iatrogenic interruption of biofilm layers. Half of each tissue sample collected from nasal fossa was treated with three cycles of 5-s spraying cryotherapy with complete thawing of the area treated between each application. Spray cryotherapy was applied with the CRY-AC-3 cryogenic Brymill (Ellington, CT) system, a device commonly used in cryosurgery for common skin disorders, which is also well suited for endonasal applications. The other sample half was left intact serving as a witness.

Histological Examination

Immediately after the nasal biopsies were harvested, the tissues were immersed for fixation in 10% neutral-buffered formalin (NBF) for 48–72 h. After transverse trimming, the nasal biopsies were dehydrated using an ethylic alcohol gradient (70%, 80%, 95%, 100%), clarified in xylene and embedded in paraffin wax.

Multiple tissue sections were cut from each paraffin block at 4 μm thickness and routinely stained with HE, Massons' trichrome (TM) and TB [29, 30]. The stained histological slides were examined by an Olympus BX41 microscope and images were obtained with an Olympus UC30 digital camera and finally processed by Olympus Stream basic.

The identification and description of bacterial biofilms was guided by the presence of characteristic morphology of microcolonies on examination by optical microscopy (clusters of small basophilic bacteria and host cells) and the presence of the surrounding polysaccharide layer. Based on their integrity and thickness, biofilms were classified in accordance with Toth et al. [30] into three groups: (1) fragmented (0–10 μm), (2) complete (< 10 μm), and (3) bulky (> 10 μm).

The aspect of the epithelium (structure, integrity, and cellular infiltration) as well as that of the subepithelial layers were correlated to the presence of microbial biofilms at each site and recorded based on local alterations. Interpretation was done by the same pathologist who was blinded in regard to cryogenic application.

Statistical Analysis

Statistical analysis was performed using the SPSS statistical program (PASW statistics for Windows, Version 20.0, Chicago: SPSS Inc. USA). Gathered data was evaluated using descriptive statistics and inferential statistics where appropriate. For all statistical tests used, $p < 0.05$ was considered significant.

Results

A total of 23 patients with bilateral CRS were included in the study, 18 patients with nasal polyps and 5 patients without nasal polyps. Four (17%) patients presented allergy to airborne particles, six (26%) patients had bronchial asthma, and two (8%) were diagnosed with ASA triad (also known as Widal or Samter's triad: nasal polyposis, bronchial asthma, and aspirin intolerance). The average Lund-Mackay score calculated was 18 (range, 3–24). Eleven (48%) patients presented a history of multiple endonasal interventions (range 1–11). Thirty-four percent of patients (8 out of 23) had visible pus at initial endoscopic examination. Table 1 summarizes the clinical characteristics of our patients.

Out of the 23 patients enrolled, only 5 (22%) had positive cultures, considering the measures taken not to contaminate the swabs. Three were positive for *Staphylococcus aureus*, *Pseudomonas Aeruginosa*, and *Escherichia coli*. Except for the latter, all cultures presented resistance to penicillins, cephalosporines, and quinolones. The presence of pus in the nasal cavity prior to surgery did not demonstrate a significant correlation with the presence of either planktonic (positive culture) ($p = 0.316$) or biofilm bacteria ($p = 0.89$). Four bacterial swabs out of the five which yielded a positive culture were positively related to the presence of biofilms.

Table 1 Clinical characteristics of patients with CRS

	Number of patients	% patients
Characteristic	23	
Males	14	60
Mean age (SD)	53.7 (14.6)	
Nasal polyps	18	78
Allergy airborne particles	4	17
ASA triad	2	22
Lund-Mackay score (range)	18 (3–24)	
Previous ESS	11	48
Topical steroid	7	30
Pus	8	34
Positive culture	5	21.7

On histological examination, 74% ($n = 17$) of patients with CRS enrolled in the study showed evidence of biofilms (Fig. 1). There were no significant differences in the results obtained through the two methods (HE stains and TB stains) ($p > 0.05$). The latter exposed clearly polysaccharide-rich material consistent with biofilm matrix (strongly positive for toluidine blue), accompanying granular material (bacteria) (Fig. 2). We found no major discrepancies between the two approaches.

Histopathological examination of the rhinosinusal mucosa revealed inflammatory changes with eosinophilic, lymphocytic (LPC), and polymorphonuclear (PMN) infiltration of the stromal layer in all cases (Fig. 1). The presence of biofilms overlying the mucosa was strongly associated with adjacent pathological alterations due to the subepithelial inflammatory reactions: epithelial desquamation, squamous cell metaplasia, and LPC or PMN infiltrations of the subepithelial layer. Biofilms not related with these modifications were scarcely seen. On statistical examination, the presence of biofilms was also associated with the presence of nasal polyps ($p < 0{,}007$), higher CT scores ($p < 0.01$), and multiple surgical interventions ($p < 0.05$). Comparison of biofilm-positive and biofilm-negative patients is presented in Table 2.

After spray cryotherapy was applied on the tissue samples, biofilms were identified in five cases (Table 3). They also showed particular histopathological aspects: they were more frequently fragmented, the matrix was lax containing various vacuoles, and they also seemed to present less adherence to the epithelium (Fig. 3). The stroma also presented important vacuolization due to the cryogenic trauma.

Discussions

Chronic sinusitis is a complex disease that represents a real burden for the individual as well as for the medical system.

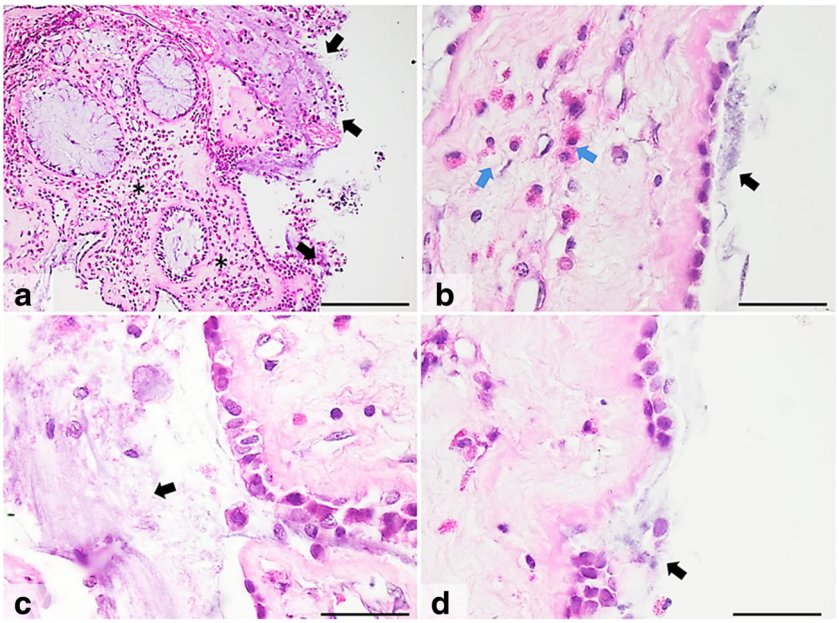

Fig. 1 The histopathological examination of the nasal mucosa. Hematoxylin-eosin staining, obx20X for image (**a**) (scale = 200 μm); ob×100 for image B, C and D (scale = 40 μm). **a** Polypoid mass with mucus gland hyperplasia, epithelial metaplasia, infiltrated with eosinophils (asterisks), and basophilic supramucosa film, rich in leucocytes (black arrows). **b**–**d** Respiratory epithelium of the nasal mucosa, covered by a basophilic, reticular mass, rich in cellular debris and granular formations—bacteria, cocci indicating the presence of biofilms (black arrows). Subepithelial edema, numerous eosinophils is emphases with blue arrows in image (**b**)

Treating these patients is often a challenge for ENT doctors because of its multifactorial pathophysiology. Intense ongoing research is undertaken to better understand the many sides of this condition and come up with new therapeutic strategies. It is now believed that the chronic inflammatory process is due to the intricate interaction between environmental and host factors. The presence of biofilms colonizing the rhinosinusal mucosal surface seems to be an explanation for the initiation and perpetuation of this pathology, especially in recurrent cases of CRS. In clinical practice, there is a significant subpopulation of patients with persistent sinus disease regardless of rigorous treatment regimens including prolonged antibiotic therapy, corticoids, allergy therapy, and surgery [31]. The motive for treatment failure could be related to a series of factors like defects in mucociliary clearance, altered local host innate immune response, or maladaptive T helper 2 lymphocytes (TH2) tissue inflammation, resulting in host-microbial imbalance and biofilm formation that will further maintain the chronic inflammatory processes [32•]. The effectiveness of several agents has been studied with promising results including post-surgery local ofloxacin, mupirocin, manuka honey, detergent-like agents, photodynamic therapy, and low-frequency ultrasound [25•]. Nonetheless, evidence concerning optimal management for recalcitrant sinusitis due to biofilms is currently lacking.

We have identified the presence of biofilms in 74% (n = 17) of cases according to data published so far [12••, 32•, 33–36]. Identification was done on histopathological hematoxylin-eosin stains by optical microscopy (OM). The novelty of this study is that we also used toluidine blue stains, which showed strong evidenced of biofilm presence (Fig. 4) and correlated with the HE findings. Although some authors promote more complex approaches of highlighting the presence of biofilms like scanning electron microscopy (SEM), transmission electron microscopy (TEM), fluorescent in situ hybridization (FISH), and confocal laser scanning microscopy (CLSM) [11, 12••, 13], we believe that HE or toluidine blue stains are similarly as effective, but faster, cheaper, and more accountable when compared to these former procedures.

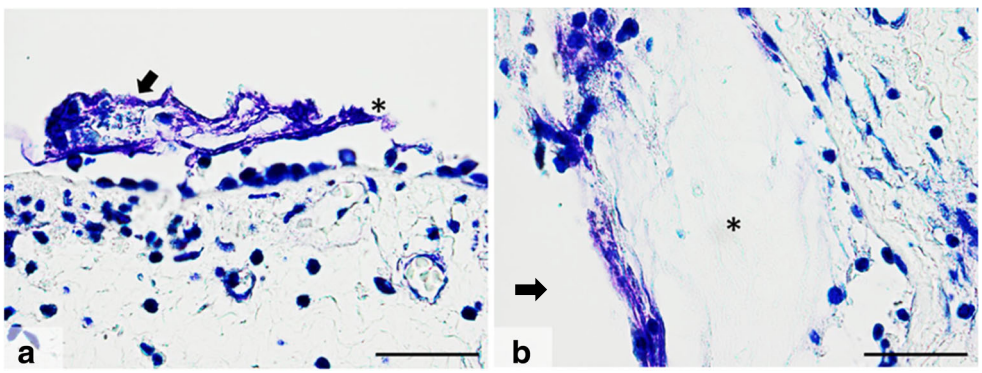

Fig. 2 Histopathological examination of the rhinosinusal mucosa. Toluidine blue staining, ob × 100 for images (**a**, **b**) (scale = 40 μm). Images (**a**, **b**) indicate the polysaccharide-rich material (asterisk marked, strongly positive for toluidine blue) associated with bacterial biofilm represented by the granular material on the mucosal surface (bacteria / cell debris) is indicated by black arrows

Table 2 Comparison of biofilm and nonbiofilm patients

	Biofilm present $n = 17$ (%)	Biofilm absent $n = 6$ (%)
Nasal polyps	16 (94%)	2 (33%)
Lund-Mackay median (range)	18 (16.0–24.0)	11.5 (3.0–17.0)
Pus visible at endoscopy	6 (35%)	2 (33%)
ESS > 1	11	–
Average no. of previous ESS	2.34	–
Positive bacterial culture swab	4 (23%)	1 (16%)

Biofilms can be formed by individual species of bacteria/fungi or they can be polymicrobial. Although many studies have focused on the identification of the distinct infectious agents in their structure, the presence of biofilm itself is thought to be the most important factor in the pathogenesis of CRS, withholding prognostic value [32•]. We have also sought to detect biofilms as a unit on the mucosa surface; microbiological identification of specific bacteria requires further special culturing techniques or FISH analysis with species-specific oligonucleotide probes [11, 12••, 13].

The presence of visible intranasal pus prior surgery was not shown to correlate with positive cultures or biofilm identification. Swabs were positive in 21.7% of cases, thus microbiological testing in current medical practice is not reliable by this method alone. This outcome is consistent with recent research that suggests that bacteria organized in biofilms cannot be cultured and isolated by conventional microbiological protocols [31] probably due to their slow metabolism and low growth rate. Therefore, diagnosis is often uncertain. More so, paranasal sinuses are not sterile in healthy individuals and microbiological testing is usually performed only in complicated cases [17].

We found patients with biofilms to have significant local inflammatory cells infiltrate and tissue destruction, directly opposed to these bacterial structures. They presented more often nasal polyps ($p < 0.01$), significantly higher Lund-Mackay scores ($p < 0.004$), and multiple ESS procedures ($p = 0.043$). In previous published studies, biofilms have been strongly associated with treatment failure and persisting symptoms [37]. Our findings correlate with published data, supporting the important role of biofilms in CRS pathology, and particularly in sustaining the inflammatory process in refractory cases.

Bacterial biofilms are characterized by extremely high resistance against antibiotics and host immune reactions. This is owed to their extracellular matrix formed of polysaccharides, proteins, and nucleic acids that acts like a physical barrier in the way of antibiotics, superoxides, immunoglobulins, and opsonins diffusion [31]. Therefore, new therapeutic strategies are needed to overcome this challenging problem. We studied for the first time the effect of spray cryotherapy on biofilms in CRS, in an in vitro model. Cryotherapy acts in the superficial layer where it instantly freezes the tissues and induces cell death with minimal local adverse reactions. Albu et al. [27] showed in a recent study that intraoperative cryotherapy is a safe and efficient procedure that improved postoperative mucosa healing and middle meatus appearance, without noticeable adverse side effects. After spray cryotherapy application on the rhinosinusal tissue surface, we noticed a significant reduction of biofilm presence; they could only be identified in 8 out of 17 (47%) biofilm-positive patients ($p < 0.001$). We also noticed important alterations in their structure where they were present, including fragmentation, matrix vacuolization, and separation from the mucosa.

The present study represents a first step in demonstrating that spray cryotherapy might be effective in the eradication of pathogenic antibiotic-resistant biofilms commonly associated with CRS. The utility of spraying cryotherapy is sustained by a previous study on animal model with maxillary antrostomy patency, being demonstrated to be a relevant model for CRS [2••]. There are no reported serious side effects with this method, except for the occurrence of intraoperative events in the treatment of tumoral airway stenosis, non-related to the direct application site [38••]. The complication can be reduced by

Table 3 Features of rhinosinusal mucosa before and after cryotherapy

	Native rhinosinusal mucosa $n = 23$ (%)	Cryothreated rhinosinusal mucosa $n = 23$ (%)	*P* value
Biofilm present (hematoxilin-eosine)	17 (74%)	8 (34.7%)	< 0.001
Class of biofilm			
Fragmented (0–10 μm)	3 (13%)	6 (26%)	< 0.005
Complete (< 10 μm)	6 (26%)	2 (8.6%)	0.346
Bulky (> 10 μm)	8 (34.7%)	–	0.43
Toluidine Blue stain—biofilm presnt	16 (69.5%)	8 (34.7%)	< 0.001
Fragmented (0–10 μm)	3 (13%)	5 (21.7%)	0.02
Complete (< 10 μm)	8 (34.7%)	3 (13%)	0.56
Bulky (> 10 μm)	5 (21.7%)	–	0.09
Biofilm vacuolation	–	5 (21.7%)	0.033

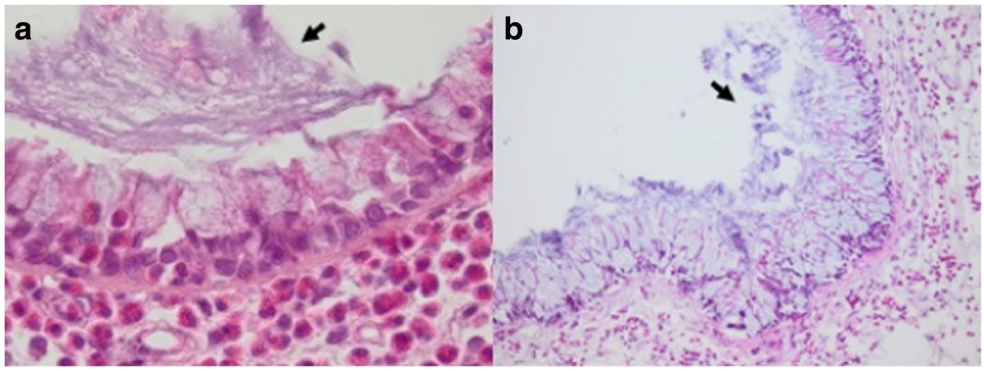

Fig. 3 Histopathological examination of the rhinosinusal mucosa after cryotherapy application. Hematoxylin-eosin staining, ob × 100 (40 μm scale). **a**, **b** Indicate respiratory type epithelium with mucus-producing cells hyperplasia, the subepithelial layer is predominantly edematous and infiltrated by eosinophils. Bacterial biofilms overlying the epithelium (black arrow) presenting vacuoles inside their structure (**a**) and fragmentation (**b**)

assuming strict protocols to maximize passive venting and to permit for adequate oxygenation [39]. The main issue is related to long-term follow-up studies that are lacking considering the possibility of unwanted changes in the mucosa.

This outcome could be due to the breakdown of the extracellular matrix or to the interruption of biofilm attachment to the sinus epithelia and the mechanical removal thereof. Further studies to evaluate the effectiveness of this new therapeutic approach are needed, including a long-term prospective human clinical trial to study the bacterial growth and behavior on an extended period of time.

Conclusion

Understanding the pathology of CRS and finding the optimal treatment remains a work in progress, particularly when it is related with high variation among the patients. In spite of many recent advances in this domain, there still is an important part of subpopulation of CRS patients who suffer from recalcitrant disease as effect of biofilm formation.

Biofilms are thought to be an important exogenous agent in sustaining the ongoing sinus inflammatory reaction, strongly associated with treatment failures and persistent symptoms. This study indicates that cryotherapy is a potential therapeutic adjuvant that targets microbial biofilm in CRS. Bacterial biofilms were removed in 70% of the cases and presented important injury in the remaining 30%. Therefore, this quite simply can be done as an outpatient procedure; the main issue is to minimize the injuries and to reduce local inflammation. These results may provide the basis for a prospective human study investigating the efficacy and safety of this therapeutic modality on a long-term follow-up, based on the fact that was not registered important adverse effects of this approach in

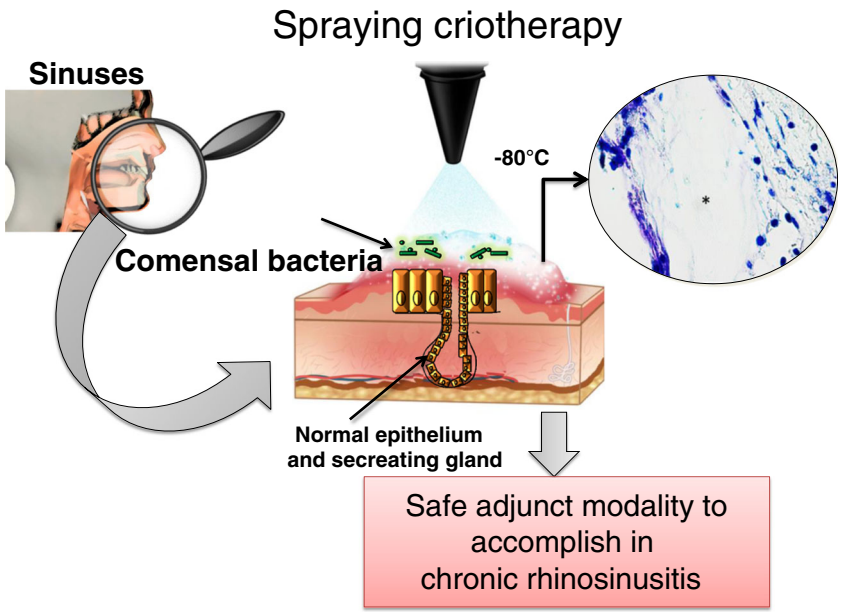

Fig. 4 Summary of application of spraying cryotherapy in the management of CRS

CRC. Although its efficacy demonstrate the need to be additionally validated for CRS, it may be notably applicable for those cases that the classically treatment failure.

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Compliance with Ethical Standards

Conflict of Interest None to be declared.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Abbreviations *CRS*, chronic rhinosinusitis; *ESS*, endoscopic sinus surgery; *HE*, hematoxylin-eosin (HE) and; *TB*, toluidine blue; *FESS*, functional endoscopic sinus surgery; *CT*, computerized tomography; *ENT surgeon*, Ears-Nose-Throat surgeon; *TM*, Massons' trichrome; *CLSM*, confocal laser scanning microscopy; *LPC*, lymphocytic; *PMN*, polymorphonuclear; *TH2*, T helper 2 lymphocytes; *SEM*, scanning electron microscopy; *TEM*, transmission electron microscopy; *FISH*, fluorescent in situ hybridization

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- •• Of major importance

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