Models for the Study of Nasal and Sinus Physiology in Health and Disease: A Review of the Literature

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Objective: Chronic sinusitis is a very common yet poorly understood medical condition with significant morbidity. Hence, it remains an entity that is difficult to treat with unsatisfactory outcomes of current management options. This necessitates research into the etiology and pathophysiology of the condition to enhance our knowledge and the therapeutic options. Unfortunately, this kind of research is not always feasible on human subjects due to practical and ethical limitations. Therefore, an alternative model that simulates the disease had to be found in order to overcome these limitations. These models could either be in vivo or in vitro. The aim of our review is to summarize the research findings and key discoveries of both in vivo and in vitro models of chronic sinusitis that have enhanced our understanding of the condition today and have paved the way for the future research of tomorrow.

Data Sources: PubMed literature review.

Methods: A review of the literature was conducted to identify the main successful in vivo and in vitro models for chronic sinusitis.

Results: Creating a successful model for chronic sinusitis is no easy task. Over the years, both in vivo animal models and in vitro tissue culture models were proposed, with each model having its accolades and pitfalls, with the ideal model remaining elusive to this day. However, advancing three-dimensional cell culturing techniques seems to be a promising new way to find a more accurate model.

Conclusion: None of the current models is perfect for a thorough study of chronic sinusitis. However, three-dimensional cell cultures have the potential to bridge the gap between in vivo and in vitro studies.

Key Words: Chronic rhinosinusitis, sinusitis, experimental models, in vivo models, in vitro models, and cell cultures.

Level of Evidence: NA

INTRODUCTION

The respiratory tract epithelium is a major point of interaction between a living organism and its environment; therefore, it has protective and adaptive functions that allow it to serve as a barrier to environmental elements that may be harmful to the living organism. However, the airway epithelium is thought to play a very important role in the etiology of airway disorders and diseases. Chronic rhinosinusitis (CRS) is an important example of such diseases. It is one of the most common, but least understood, medical conditions. Despite an estimated prevalence of 15.7% among the general population in the United States, CRS remains a disease recalcitrant to current management options.[1] Despite its low efficacy, the medical management of CRS has changed very little over the years, and even in the face of rapid technical advances in the field of surgery, the surgical options for CRS only offer a tamponade solution, with a high incidence of postoperative recurrence.[1,2] Thus, an improved understanding of CRS could potentially offer better solutions for the management of the disease. Over the years, different models have been proposed toward improving our understanding of the pathophysiology of CRS. Each model has its pros and cons. These can be broadly classified into in vivo and in vitro models. This review examines the current literature on the various models proposed for the studying CRS and summarizes the research findings in this field.

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Pathophysiology of Rhinosinusitis

The most basic definition of rhinosinusitis is inflammation of the lining membrane of the nose and paranasal sinuses. The inflammation can be triggered by local or systemic factors and could be infectious or noninfectious. Local factors include ostial narrowing, ciliary dysfunction, and mucosal injury leading to impaired mucociliary drainage and the subsequent overgrowth of pathogenic microorganisms, whereas the systemic factors are mainly related to immune dysfunction or

allergy.[3] Clinically, rhinosinusitis can be classified according to the duration of symptoms into acute, subacute, and chronic forms. According to the American Academy of Otolaryngology–Head and Neck Surgery (AAO–HNS), symptoms of rhinosinusitis lasting less than 4 weeks are classified as acute rhinosinusitis, while those lasting more than 12 weeks is classified as CRS. The most recent guidelines published by the AAO–HNS do not recognize subacute sinusitis as a distinct clinical entity anymore, but it was defined by symptoms lasting more than 4 weeks but less than 12.[4,5] Both acute and chronic forms of rhinosinusitis could be regarded as two ends of a spectrum. Similarly, there's a great degree of overlap in the pathological features of both diseases. However, acute sinusitis is distinguished from chronic sinusitis by the type of cells and cytokines involved in the inflammatory milieu. Acute sinusitis, irrespective of whether caused by bacterial or viral causes, is characterized by increased neutrophils, interleukin (IL)-1α, IL-6, and IL-8. This condition is termed as "infectious inflammation."[6] On the other hand, while chronic rhinosinusitis could be instigated or caused by an infection, the accompanying inflammatory response is much more complex and often no evidence of an infection could be traced, thus it is dubbed as a "non-infectious inflammation." However, CRS secondary to a chronic dental apical abscess is a notable exception as the source of the infection could be traced. CRS can be sub-classified clinically to CRS with polyps (CRSwNP) and CRS without polyps (CRSsNP). Pathologically, CRSwNP and CRSsNP also differ in the type of cellular infiltrates and inflammatory mediators. Classically, eosinophils are frequently detected in the mucosa of polyp samples obtained from patients with CRSwNP. However, CRSsNP is characterized by glandular hyperplasia.[7,8] However, both types of CRS are characterized by a predominantly lymphocytic infiltrate and paucity of neutrophils in comparison to acute sinusitis.[9] Furthermore, patients who have CRS with an allergic component have high levels of T-helper 2/T-helper 1 ratio of lymphocytes in comparison to those who have CRS without allergy. CRS without allergy is a mixed T-helper 1 and 2 response.[10,11] Thus, CRS is a very complicated pathological process that is poorly understood. Therefore, the development of tools that would facilitate a better understanding of this process, and how to treat it, remains imperative.

In Vivo Models

Although many researchers have attempted to investigate the pathophysiology of CRS in humans, their investigations have been limited by several key problems. Firstly, the availability of representative tissue samples from humans is low. Secondly, the variability is high and the reproducibility low because of the variability in the external in vivo conditions and the interindividual variability. Variability in the etiology, microbiology, genetics, and extent of inflammation between different subjects have made the usefulness of the assessment of individual responses to microbes very limited. The use of animal models eliminates most of this variability and provides adequate tissue samples.

However, to date, the ideal animal model has yet to be determined.[12–15] The most extensively studied animal for sinusitis is the rabbit, but in recent years, murine models have been explored. We will review the key studies conducted on these two animal models.

The rabbit model for sinusitis

The rabbit is often promoted as the prototypical experimental animal model of rhinosinusitis. This is mainly because of the similarities between its sinus anatomy and its immune responses and those of human beings (Fig. 1) as well as the favorable size of its sinuses and relative ease of access to them.[15] In 1941, Hiding introduced the first rabbit model of sinusitis by performing sinus surgery on the rabbit. He performed external-approach antrostomy at four different sites. The locations were as follows: through the natural ostium, just adjacent to it, along the sinus floor, and at a distal location far from the ostium. He noted that five of six rabbits developed sinusitis when antrostomy was performed through the ostium or adjacent to it, while only one of the six rabbits developed sinusitis on antrostomy performed in other locations far away from the ostium.[16] After Hiding's discovery, very little was done until 40 years later. In 1981, Maeyama et al. induced allergic sinusitis in rabbits by repeatedly injecting them with *Staphylococcus aureus* following previous sensitization with egg-white albumin.[17,18] Shortly afterwards, a group of researchers from the Karolinska Institute in Sweden developed their model by both obstructing the ostia with tissue glue and injecting the maxillary sinus with an aliquot of pathogenic bacteria. The result was a 100% reliable method for inducing sinusitis in rabbits.[19,20] Their model has been extensively applied, particularly in studies on the etiology and pathogenesis of sinusitis and polyposis. Using their model, Westrin et al. studied the histopathology and histochemistry of the infected inflamed mucosa and mucus, and observed an increase in glandular epithelium, with hyperplasia of goblet cells and lactic acid build-up in the mucus. They also examined the immune response to infection and noted the development of antibodies to the pathogenic bacteria, with titers correlating with the severity of the infection. Moreover, they were able to create a more severe, longer lasting infection by injecting *Bacteroides fragilis*, which allowed them to study the effects of inflammation over a longer period of time.[21–24] Min et al. used this model to study the effect of opening the obstructed ostium and found that doing so would significantly reduce the severity of the inflammation. They also studied the effects of systemic antibiotics and topical delivery of antibiotics to the sinuses after relieving the obstruction. They concluded that topical antibiotics were superior to systemic antibiotics in reducing the inflammation.[25,26] Bende et al. further examined the effects of topical medications on the sinuses after relieving the obstruction and discovered that the infection worsened with the use of topical decongestants. They attributed this result to decreased mucosal perfusion.[27]

While the rabbit model served as the basis of great discoveries, it was not without flaws. Hinni et al.
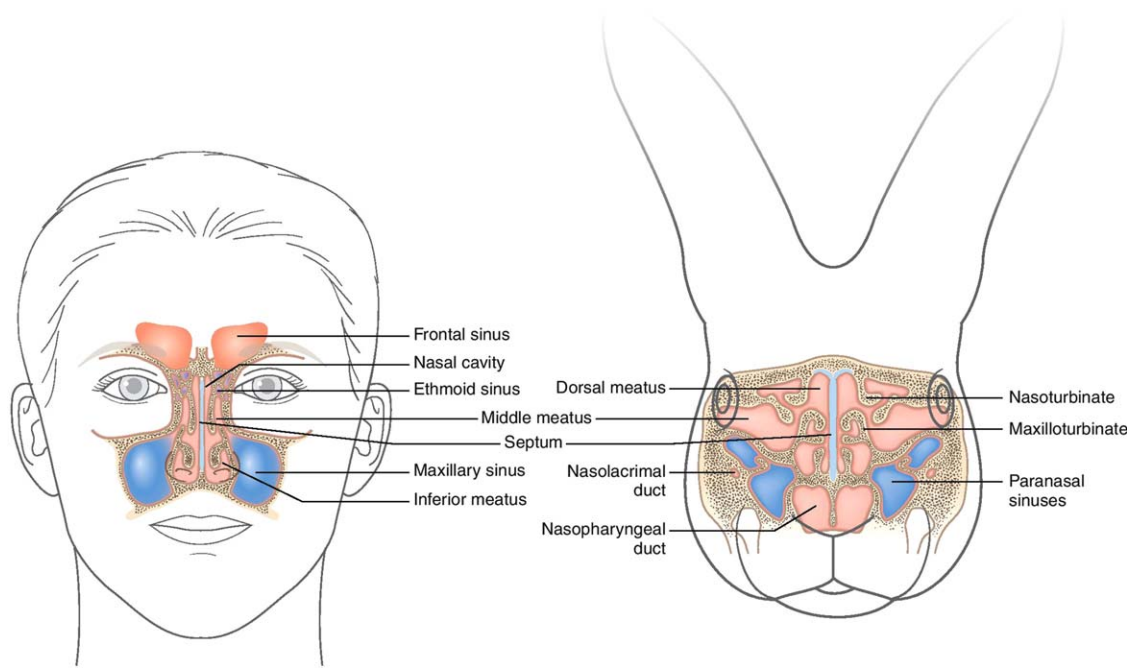
Fig. 1. Comparison of human and rabbit sinus anatomy.

criticized the model as being too physiologically disruptive. They argued that mucociliary function and capillary perfusion are violated by surgically opening the sinuses. They developed an alternative method of occluding the natural ostia through the nasal roof, thereby eliminating the need for opening the sinuses. This model was also reliable in inducing sinusitis.[28] However, infection from both models is usually limited to the maxillary sinus. In humans, infection in the anterior ethmoid sinuses is believed to cause inflammation around the ostium of the maxillary sinus and the infection in the maxillary sinus is considered the result of infection spread from the anterior ethmoids. This theory emphasizes the importance of the ethmoid sinuses in the pathogenesis of sinusitis.[29,30] Moreover, complete ostial obstruction in humans would result in the formation of a mucocele, which is the accumulation of mucous within the sinuses, or mucopyocele, which is formed in the presence of infection. Complete ostial obstruction would rarely result in classic sinusitis in humans.[31–33] In 1997, Steven Marks dubbed the previous models as being "sinogenic," which implies that the inflammation originates from the obstruction of drainage and bacterial inoculation of a specific sinus and therefore precludes involvement of other sinuses. His theory is that in reality, sinus infection typically results from "rhinogenic" sources, eg, inflammation from allergies or viruses. In turn, this leads to dysfunctional cilia, accumulation of mucous, changes in gas composition within the sinuses, and subsequent bacterial overgrowth. Furthermore, he pointed toward a major drawback of sinogenic models, wherein the hypoxic sinus is injected with an overwhelming amount of bacteria, resulting in a very brisk, nearly necrotizing infection. Thus, the response could not be graduated and the severity of the infection masks many important subtle effects that are only appreciated when the inflammation is less intense and more chronic. Therefore, he presented an alternative "rhinogenic" model that eliminated the need for surgical manipulation, allowing a uniformed involvement of the sinuses and permitting a better understanding of the gradual natural course of sinusitis. He created his model by placing a polyvinyl acetate absorbent sponge (Merocel) impregnated with bacteria through the nostrils inside the nasal cavity. Sinusitis followed a few days after the insertion by pathological examination.[34] On the basis of Marks's milestone work, Kara et al. and Beste et al. adopted the rhinogenic model and performed serial computed tomography (CT) scanning of the sinuses after the placement of the Merocel sponge and for up to 30 days after removal of the sponge. They found that although spontaneous resolution of sinusitis was not achieved unless antibiotics were used, the intensity of inflammation decreased over time from week 2 onwards.[35,36]

Indeed, one issue common to all the models explored so far was their inability to simulate CRS, which by definition requires more than 12 weeks of persistent inflammation.[1] Liang et al. modified Marks's model to induce sinusitis that is more than 12 weeks in duration to better mimic CRS. They achieved this by soaking the Merocel sponge with phorbolmyristate acetate (PMA), a biologically active compound capable of inducing chronic inflammation. Although chronic inflammation was induced in the modified rhinogenic model by Liang et al., no polyps were observed macroscopically or microscopically.[37] More recently, Sejima et al. proposed an alternative method to induce eosinophilic polyps in a sinogenic model. They sensitized the rabbits by subcutaneous injection of ovalbumin (OVA) and then surgically occluded the natural sinus ostium and injected valine-
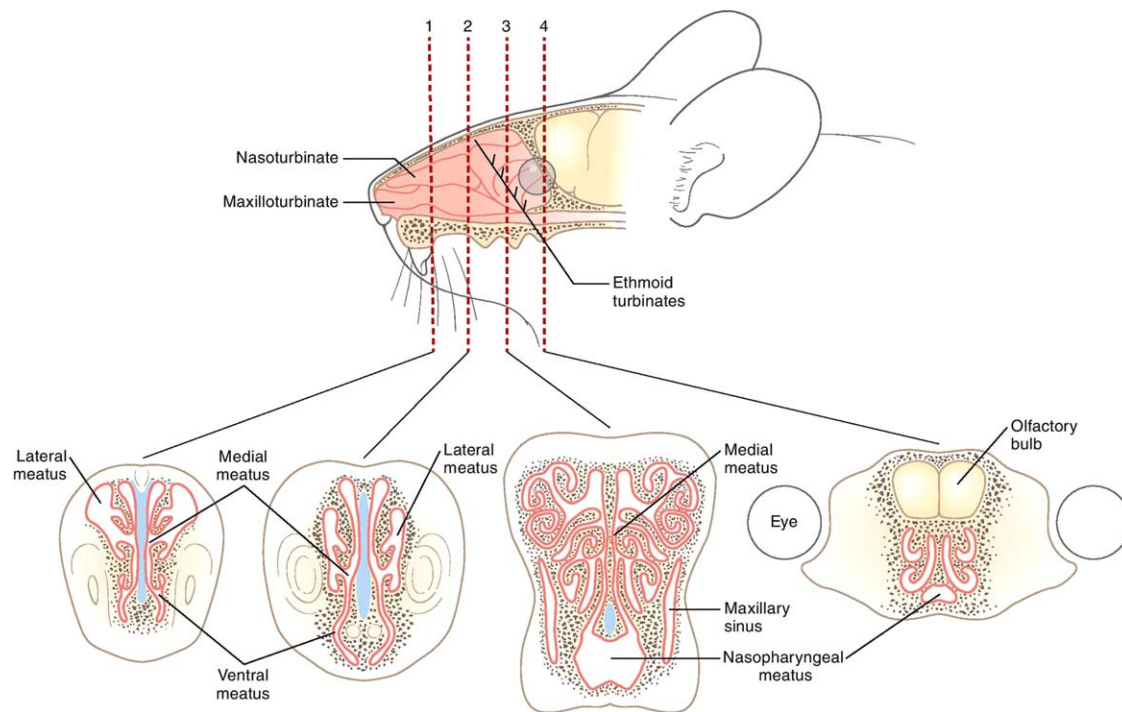
Fig. 2. Murine sinus anatomy: a sagittal section of the skull and the corresponding coronal sections through the nasal cavity.

glycine-serine-glutamic acid (VGSG) or poly-l-arginine (PLA) into the sinuses. They observed the development of eosinophilic polyps with both injectables, although polyps in sinuses injected with PLA tended to be larger.[38]

In summary, although no ideal model has been established thus far for sinusitis especially for CRS, we have come a long way since the first experimental rabbit model of sinusitis developed by Hiding. The rhinogenic model of sinusitis offers many advantages over the sinogenic model in that the former is more practical, it eliminates the need for invasive surgery, and is easier to establish and reproduce. In addition, it can be used to study new therapeutic modalities. However, it is yet to demonstrate its utility in studying polyps in CRS.

The mouse model for sinusitis

Although the sinus anatomy of mice is not similar to that of human beings, the respiratory epithelium is the same (Fig. 2). Therefore, epithelial remodeling, inflammatory cell infiltration, and collagen deposition can be evaluated in mice during experimental conditions. Murine models did not gain much popularity in the past, but in recent years they have been investigated as an alternative option to rabbits due to the ability to manipulate them genetically, and the availability of a wider range of reagents and antibodies for the detailed study of inflammation in murine models. Bomer et al. were the first to develop a murine model of acute sinusitis by intranasal inoculation with *Streptococcus pneumoniae*.[39] Subsequently, Won et al. used this model to test the effects of antibiotics on infection and inflammation. They found that by treating the mice with trimethoprim-sulfamethoxazole injections, there was a dramatic decrease in the number of *S. pneumoniae* colonies and the number of neutrophils on microscopic analysis.[40] While this model was successful in inducing bacterial sinusitis, the inflammation did not last beyond 2 weeks, and therefore, it is categorized as a model for studying acute infection.[39] Mice could also be useful as models for CRS. Jacob et al. were able to induce a response over a longer period of time. In their experiment, they surgically placed a Merocel sponge alone or impregnated with *Bacteroides fragilis* in the nasal cavity of the mice. They observed microscopic inflammatory changes similar to those observed with CRS after four weeks of the placement of either type of Merocel sponges; however, the inflammation was more severe with the one impregnated with *B. fragilis*.[41] Wang et al. further investigated the immunological cascade occurring in CRS and the involved immune mediators and cytokines.[42] Mice were also used to study viral, fungal, and allergic sinusitis. Ramadan et al. and Ahn et al. used intranasal inoculation with reoviruses or *Aspergillus/Alternaria* fungi to study the immune response to viruses and fungi, respectively.[43,44] Lindsay et al. developed a mouse model of chronic eosinophilic rhinosinusitis using *Aspergillus fumigatus*. Interestingly, *A. fumigatus* has been implicated in the pathophysiology of both chronic eosinophilic rhinosinusitis and allergic fungal sinusitis, making their model useful for studying either or both conditions.[45] Moreover, because sinus surgery is often complicated by synechiae secondary to mucosal injury and the subsequent healing process, Tansavatdi et al. took the model developed by Lindsay et al. further by studying the effects of mucosal injury on the

| TABLE I. |||||
| Abilities and Limitations of In Vivo Animal Models. |||||
The rabbit model		The mouse model	
Abilities	Limitations	Abilities	Limitations
• Can illustrate the histopathological features of sinusitis. • Can be used to study the microbiology of sinus infections. • Defined the role of ostial obstruction and microbial inoculation in the development of the disease.	• Inadequate for studying chronic inflammation and the development of polyps. • Relies on ostial obstruction to induce sinusitis. • Limited reagents and antibodies for the detailed study of the immune cascade.	In addition to the abilities of the rabbit model, mice: • Can be used to study the genetics of sinusitis. • Can be used to study the immune cascade in response to infection with much greater detail.	• Unrepresentative sinus anatomy. • Higher risk of complications from manipulation than rabbits.

chronically inflamed sinus mucosa effectively mimicking injury during surgery. They wounded the mucosa using a Rosen needle then examined the histologic and gene expression effects of the inflammatory response after wounding. They also compared it to wounding the non-inflamed sinus mucosa in normal mice. As expected, inflamed mucosa showed a stronger response to the needle trauma, therefore, had a higher potential for scarring during wound healing.[46] An allergic sinusitis model in mice was established by Kim et al. They induced allergic inflammation in mice with ovalbumin (OVA), an allergenic protein found in chicken egg whites. This model was created by instilling 3% OVA in the nasal cavity of the mice three times a week for 8 weeks to induce an eosinophilic reaction and subsequent inflammation. They also succeeded in inducing nasal polyps with the same model by adding *Staphylococcus aureus* enterotoxin B (SEB), proving that SEB plays a role in in the pathogenesis of nasal polyps. To induce nasal polyposis, 3% OVA plus 5 ng of SEB were instilled in the nasal cavity once a week for 8 weeks. It is hypothesized that OVA sensitizes the nasal mucosa allowing SEB to cause a more intense eosinophilic reaction that leads to the formation of nasal polyps.[47] Polyp formation in the murine model was identified by morphology during microscopic examination. Nasal polyps were characterized as an edematous stromal connective tissue that is more elevated than the surrounding mucosa and is infiltrated by eosinophils.[48] More recently, Kim et al. created a model for recalcitrant allergic CRS by challenging the mice with OVA and *Aspergillus* protease, which is known to trigger allergic reactions in mouse lungs.[49] However, their original model using OVA and SEB has allowed many great discoveries about the pathophysiology of CRS.[50–59] In some transgenic strains of mice, small modifications to the concentration, dose, and frequency are required to induce inflammation and nasal polyposis. For example, Lee et al. used 6% OVA three times a week instead of 3% three times a week, and 10 ng of SEB three times a week instead of 5 ng once a week. The higher stimulation was needed because the strains they used had an attenuated airway hyper-responsiveness to allergens compared to the other strains. The discovered that certain transcriptional factors played a role in the pathogenesis of nasal polyps by using these transgenic mice.[57] In addition to all the

previous studies, cystic fibrosis–associated sinus disease is another domain that could be potentially investigated using genetically altered mice.[60] However, despite all the pathophysiological insights gained from the OVA model, some scientists doubted its accuracy in representing allergic CRS. That is mainly due to the fact that OVA is a food allergen.[61] On that basis, Khalmuratova et al. developed a novel murine model using house dust mite (HDM). House dust mite is an airborne allergen, and is a common cause of respiratory allergic diseases. Therefore, it is thought of as a more suitable agent to induce allergic CRS. Interestingly, this novel model also showed significant mast cell recruitment, a known feature of allergic rhinosinusitis, that was not previously exhibited by other models.[62]

In summary, despite the troublesome manipulation of mice due to their size, different anatomy, and increased possibility of complications such as aspiration and pneumonia, they could serve as an alternative to rabbits. They are superior to rabbits in studying sinus disease at both genetic and molecular levels. Furthermore, the ethics of animal experimentation oblige us to use the least sophisticated animal species whenever possible. Table I. summarizes the abilities and limitations of both animals as a model for sinusitis.

In Vitro Models

While in vivo animal models have proven to be excellent tools for studying the genetics and pathophysiology of sinus disease, they most certainly have some limitations. They are poor predictors of therapeutic response in humans. In fact, this translational disparity is the entire basis of clinical trials. Humans react differently to drugs than animals, and that is why many drugs fail clinical trials despite promising results in animals.[63–65] This necessitates the development of in vitro cellular models of disease that enable the cultivation of more reliable data in predicting how the human body will react to a drug. Human airway epithelial cell cultures offer an excellent in vitro model system for the study of the epithelium in the etiology of sinus diseases as well as the underlying mechanism of inflammation and hyper-responsiveness. Table II compares in vivo with in vitro models. Epithelial cells could be cultured in many ways: organ cultures, tissues explant cultures,

Al-Sayed et al.: Models for Sinonasal Physiology and Pathology

In vivo models		In vitro models	
Abilities	Limitations	Abilities	Limitations
• Provides a good overview of the inflammatory process. • Better illustration of the dynamic immune response to microorganisms. • Good for studying the genetic causes for sinusitis.	• Cannot predict individual cell response during inflammation or response to therapy. Therefore, doesn't translate human response accurately. • Chronic inflammation is difficult to produce. • Has a great ethical burden.	• Can measure individual cell response. Therefore, more accurately translates to human responses. • Chronic inflammation can be achieved reliably. • Achieves the three R principle of refinement, reduction, and replacement.	• Depends on the technique of culturing. • Can't account for the dynamic immune response that occurs during inflammation.

monolayer cells cultures, and the more advanced three-dimensional (3D) cultures. In addition, nasal epithelial cell lines such as RPMI 2650 have been developed and used to study CRS. We will review all the methods and techniques used to establish in vitro models of sinusitis.

Organ cultures and tissue explant cultures

Organ culture refers to the culturing of all cells within an organ while preserving normal structure and cellular interactions. Explant cultures are prepared by the culturing of a tissue sample explanted from an organ. Both methods have been used to establish CRS models. Organ cultures have been used extensively for studying lung diseases with notable success in growing airway models in vitro.[66–69] However, their applicability to recreate the nasal and sinus mucosa is limited by the histological and structural complexity, the high variability and the low reproducibility.[67] On the other hand, explant cultures avoid many of the pitfalls of organ cultures and have been used to successfully grow nasal mucosa.[70,71] However, both methods have the disadvantage of the concomitant growth of non-epithelial cells, such as fibroblasts, smooth muscle cells, and endothelial cells. This cellular complexity makes it difficult to determine whether the effect of a drug is due to its direct action on the nasal epithelial cells or whether neighboring non-epithelial cells potentially give rise to unreliable data.[72]

The monolayer cell cultures

In this method, cells are isolated by enzymatic dissociation and cultured on a Petri dish coated with biologically derived matrices such as collagen, fibrin, or synthetic hydrogels. In 1985, Wu et al. proposed a method for the growth of human nasal epithelial cells in vitro from surgical samples isolated from both nasal turbinates and nasal polyps. They also developed a novel culture medium for that purpose, which was a Ham's F12 medium supplemented with insulin, transferrin, epidermal growth factor, hydrocortisone, cholera toxin, and bovine hypothalamus extract.[73] Steele et al. took the experiment further by comparing the behavior of nasal epithelial cells from rats, rabbits, and humans in vitro. They observed that by modifying the contents of the culture medium, the growth rate of the nasal epithelial

cells could be changed depending on whether they were sourced from rats, rabbits, or humans, essentially proving that human responses could differ from animal responses in experimentations.[74] Using the culturing technique developed by Wu et al., Ayars et al. created a model of cellular injury similar to that observed in rhinitis by exposing the cultured nasal epithelial cells to eosinophil granule products. They noted cellular membrane damage in response to exposure to eosinophil peroxidase, glucose/glucose oxidase, and bromide combination or eosinophil major basic protein, which are all released by eosinophils during the inflammatory response in rhinosinusitis.[75] Kenney et al. adopted another approach in investigating the inflammatory response of sinusitis by identifying and measuring the pro-inflammatory cytokines released by cultured nasal epithelial cells. They discovered that nasal epithelial cells produce IL-1α, IL-6, and IL-8 in increased quantities in response to lipopolysaccharide (a component of bacterial outer membrane).[76] Subsequent to this discovery, many investigators examined the effects of various topical medications such as antibiotics and glucocorticoids on the inflammatory mediators and the resultant inflammatory response.[77–79] However, despite these discoveries regarding cellular behavior in response to topical medications and inflammation, the monolayer cell culture method has some limitations.

Cells grown in monolayers form a flat, two-dimensional (2D) configuration. Nasal epithelial cells grown in this manner lose some of their differentiation parameters such as ciliary function and mucin production. While preservation of some of these functions are possible by altering the culture conditions with the addition of hormones, growth factors, and vitamins, these effects are short lived. By 2 to 3 weeks, almost all cells undergo a progressive morphological change into a more squamous-cell-like appearance and lose their ciliary activity and ability for mucin production (Fig. 3).[80–82] This represents a major barrier in the long-term investigations of drugs acting on the epithelium as well as ciliary function and mucin production during inflammation.

RPMI 2650 cell line culture

Cell lines are immortalized cells that are allowed to grow and divide freely in a culture medium. Cell lines
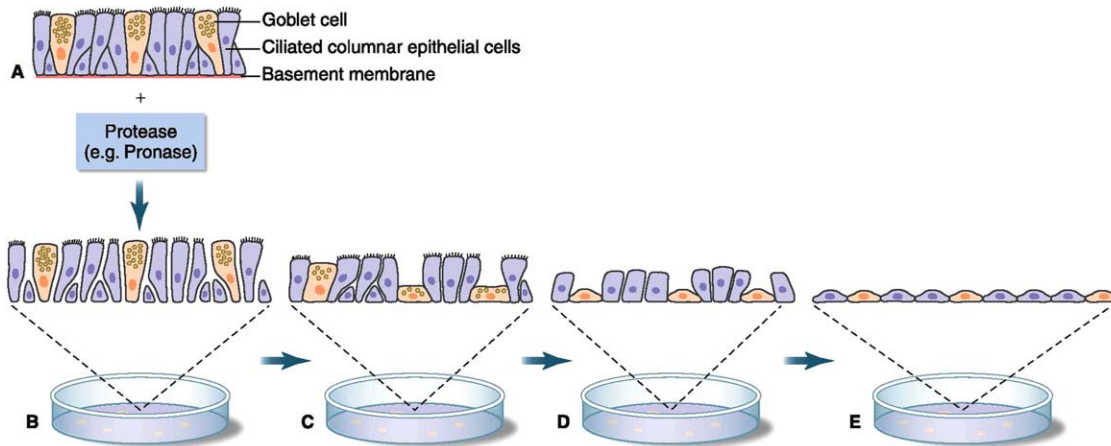
Fig. 3. Monolayer cell culture: (A) Respiratory epithelium obtained from normal or diseased mucosa. (B) Dissociated epithelial cells plated after treatment with a protease. (C) Cells lose height and starts flattening after a few days of culturing. Goblet cells starts losing its secretory capacity first. (D) Columnar cells lose their cilia and goblet cells have completely undergone squamous transformation. (E) Complete squamous transformation of all epithelial cells occurs within 2–3 weeks.

are widely used to study a disease process and many cell lines are available for various diseases.[83,84] However, the number of cell lines to study CRS is limited. At the time of this review, one cell line, RPMI 2650, is available for studies on sinonasal conditions.[85] The commercially available RPMI 2650 cell line was obtained from an anaplastic squamous cell carcinoma of the nasal septum in a 52-year-old male. The tumor cells have been shown to have a similar karyotype as nasal epithelial cells. They also showed similarities in mucous production and surface cytokeratin polypeptides.[86–88] Ball et al. sought to identify the reliability of the cell line for use as a model for CRS by comparing it to monolayer cell cultures obtained from patients with chronic sinusitis. They found that the cell line had a mixed mesenchymal and epithelial phenotype and a growth pattern different from that of the sampled epithelial cells. They attributed these differences to the neoplastic origin of the cell line. Additionally, they found that the cell line did not produce a significant inflammatory response when stimulated with tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), poly I:C, and transforming growth factor-β (TGF-β), thereby concluding that these cells are inappropriate for studying CRS.[89]

The 3D cell cultures

When cells are cultured in three-dimensional (3D) models, they closely mimic the features observed in the complex in vivo environment. The 3D cell culture models have been proven to be more realistic than traditional 2D cell cultures. They allow for a more accurate translation of study findings into in vivo applications.[90] Many techniques to prepare 3D culture cells are available, and they can be broadly classified to scaffold- and non-scaffold-based technologies. As the name implies, scaffold-based methods utilize a matrix, which in turn could be biologically or synthetically derived. These matrices are configured as a 3D interwoven mesh network of fibers to form a porous object similar to a sponge

or a simple gel. Cells attach, migrate, and fill in the interstices of this geometric network to assume a 3D configuration when cultured.[91,92] On the other hand, the non-scaffold methods utilize physical forces to make cells aggregate in a spherule, thereby assuming a 3D configuration. The spherule configuration is most commonly achieved by using micro-suspension, specially coated microplates, or a microfluidic system.[93–95] In a micro-suspension, the cells are kept hanging inside a growth medium droplet from a bottomless-well microplate, with the droplet being big enough for cellular aggregation, but also small enough such that its surface tension prevents the droplets from being dislodged during manipulation. By force of gravity, cells will aggregate and eventually assume a spherule configuration. Another technique that has an identical effect is the use of regular microplates coated with an ultra-low attachment (ULA) coating, therefore, cells aggregate and form a spherule in the middle of the well and away from the ULA-coated well walls. The third technique, the microfluidic system, introduces a continuously perfusive flowing fluid through a chamber that contains the cells. The cells are maintained in each chamber by micro-pillars, and the culture assumes a 3D configuration by aggregating due to the force of the flowing fluid, which carries oxygen and nutrients to the cells and washes cellular waste products away.[96,97]

To prevent the loss of differentiation into human nasal epithelium in monolayer cell cultures, Jorrisen et al. described an alternative method of cell culturing. They noticed that cells cultured in a monolayer fashion from surgically excised nasal polyps, lost their cilia, and assumed a squamous-like morphology after just 2 weeks in culture. However, when the same cells were cultured using a culture flask attached to a continuously rotating gyrotory shaker, they assumed a spherule 3D configuration after 3 to 5 days of rotation and can be maintained in this manner in a stationary culture thereafter. The cells they cultured by this technique maintain their morphological differentiation, typical for the respiratory
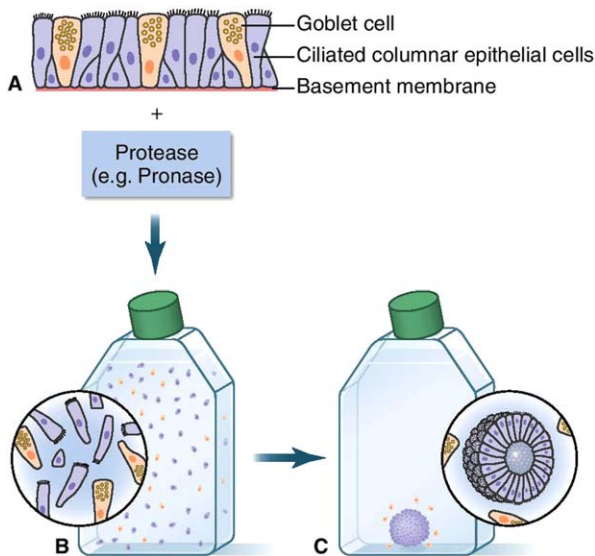
Fig. 4. 3D cell culture: (A) Respiratory epithelium obtained from normal or diseased mucosa. (B) Dissociated epithelial cells are placed in a culture flask after treatment with a protease. (C) Continuous shaking of the culture flask on a gyratory shaker allows the cells to curl up in a spherule with the apical surface of the cells pointing outwards and the basolateral membranes inwards. Cells cultured in this configuration kept their cilia even after 7 months in culture.

epithelium, even after 7 months in culture (Fig. 4). However, the major problem with this technique is that up to 50% of the cells are lost in the medium. The spherules could get disrupted and attach to the bottom of the flask or degenerate.[80] Chevillard et al. proposed a more structurally stable model by growing the nasal epithelial cells within a floating collagen gel. The growth of cells in a 3D cord-like network minimized cell loss and preserved cellular morphology as well as the mucin secretory functions of the cells.[98] Similarly, Benali et al. and Wang et al. achieved the same results by using a 3D collagen lattice.[99,100] Recently, attempts have been made to culture nasal epithelial cells in microfluidic environments. The underlying premise is that it adds a dynamic dimension to the cultured cells, thereby allowing for better replication of the dynamic in vivo environment.[97,101,102] However, microfluidic and scaffold-based cultures are much more complex than spherule-forming methods.[101–104] In addition, considerable technical advances have been made in the culturing of human epithelial cells using spherule-forming methods. Ulrich et al. modified the method used by Jorrisen et al. by performing repeated centrifugation of a nasal polyp tissue explant after treating it with pronase (a tissue-dissolving agent) in order to obtain a stable epithelial-cell spherule with intact ciliary and mucin production activity.[105] Furthermore, Castillon et al. recreated the experiment performed by Jorrisen el al. to study the electrophysiological functions of cells during spherule formation and obtained a model for cellular behavior in cystic fibrosis.[106]

An alternative method to minimize the de-differentiation of epithelial cells is to culture them at the air-liquid interface (ALI).[107] The conventional monolayer cell culturing systems depends on submerging the cells in the culturing medium to grow them. In contrast, an ALI culture has the basal surface of the cells is in contact with liquid culture medium, whereas the apical surface is exposed to air. This is achieved using specific ALI culture plates that allows "air-lifting" the epithelial cells to stand on top of a porous membrane made of either polycarbonate, polyester, polyethylene terephthalate, or polytetrafluoroethylene (Transwell).[108,109] However, despite being a suitable method to culture nasal epithelial cells for transnasal permeability and drug transport studies, it is not an ideal method to create a model for studying CRS, because the porous membrane on which the growing cells are supported is too stiff thereby potentially affecting cellular responses. Kyuh-wan et al. got around that problem by using an advanced microfluidic culturing system. They grew the nasal epithelial cells on top of a collagen or Matrigel extracellular matrix at an ALI on one side and grew endothelial cells in a conventional submerged fashion on the other, all being in a continuously perfused microfluidic chip. This complex system allowed them to ingeniously grow both the nasal mucosa and its connective tissues, thus creating a much more accurate model for studying the pathophysiology of diseases affecting the nasal mucosa [110]. However, no further studies utilizing this model have been attempted at the time of this review. Table III. summarizes the abilities and limitations of the various techniques of *in vitro* modeling.

Insights gained from in vitro models and future directions

Culturing human nasal epithelial cells requires high-fidelity. Setting up an in vitro model for sinusitis is no easy task. However, the in vitro models discussed in this review facilitated many discoveries about chronic sinusitis. Based on the hypothesis that a number of pro-inflammatory cytokines identified as possible mediators of the pathologic events in chronic sinusitis, Kenney et al. set out to prove their synthesis by cultured human nasal epithelial cells. As mentioned earlier, they utilized a monolayer cell culture model to grow the epithelial cells, which were sourced from patients with chronic sinusitis post elective surgical excision. The supernatant or lysates of the cultured cells were analyzed using an enzyme-linked immunosorbent assay (ELISA) specific for human IL-lα, IL-1β, IL-6, and IL-8 and a Northern blot for the expression of messenger ribonucleic acid (mRNA) of the same interleukins. The cultured cells produced IL-1α, IL-6, and IL-8 and their corresponding mRNAs in increased quantities. These cytokines are potent as chemotactic factors for leukocytes. This discovery implicates the nasal epithelium as a major contributor to the inflammation of chronic sinusitis.[111] Conversely, in an experiment a few years later, Ramanathan et al. explored the role of leukocytes in modulating the innate immune function of the nasal epithelium. By exposing the cultured nasal epithelial cells to IL-4 or IL-13, which are primarily a type 2 helper T cells (Th2)

	TABLE III. Abilities and Limitations of In Vitro Culture Models.						
Organ or tissue explant cultures		Monolayer cell cultures		Cell line cultures		Three-dimensional cell cultures	
Abilities	Limitations	Abilities	Limitations	Abilities	Limitations	Abilities	Limitations
• Can grow multiple types of cells simultaneously.	• Complex, highly variable, and poorly reproducible. • Difficult to isolate observation based on cellular origin.	• Can be used to study cellular responses in chronic sinusitis by measuring the mediators and cytokines produced. • Can be used to study the electrophysiological function of nasal epithelial cells during inflammation. • Observed effect can be isolated to the cultured nasal epithelial cells.	• Difficult to predict cellular behavior in terms of mucous production and ciliary activity during inflammation.	• Commercially available. • Grows rapidly.	• Inaccurate in predicting cellular behavior in chronic sinusitis thus being unreliable.	• In addition to the same abilities as the monolayer cell cultures, 3D models: • Can preserve mucous production capability and ciliary activity thus creating a more accurate model. • Versatility in culturing techniques.	

cytokines, they produced less IL-6, IL-8, and Eotaxin-3, as evidenced by ELISA and real-time polymerase chain reaction (PCR) of their corresponding mRNAs. Eotaxin-3 is a potent chemotactic factor for eosinophils, which are present in high number in nasal polyps. Moreover, they performed flow cytometric analysis of toll-like receptors (TLRs) expressed on sinonasal epithelial cells. TLRs are transmembrane receptors that interact with pathogens and signal the presence of microorganisms. TLR-9 in particular, is strongly expressed in normal nasal epithelium. Down regulation of this receptor may play a role in the pathogenesis of recalcitrant CRSwNP. Th2 cytokines were found to down regulate TLR-9 in their experiment. Thus, their findings suggest that IL-4 and IL-6 down regulate the antimicrobial mucosal immunity resulting in chronic sinonasal infection that can potentially be the instigator of CRS and nasal polyps. Additionally, they also treated the cultured cells with interferon-γ (IFN-γ), which is primarily a type 1 helper T cell (Th1) cytokine, and observed an opposite effect. Thus, suggesting that CRS is influenced by the local Th1/Th2 cytokine environment.[112] More recently, Ramezanpour et al. investigated the effects of cytokines on mucosal barrier integrity in terms of structure and function. They examined the epithelial cells cultured at ALI after exposing them to type 17 helper T cells (Th17) cytokines, as well as cytokines produced by Th1 and Th2 cells. Th17 is a subset of activated CD4$^+$ T cells involved in adaptive and innate immunity as well as allergic reactions. They act by producing IL-17A, IL-17F, IL-22, and IL-26. The measured parameters indicative of mucosal barrier function were transepithelial electrical resistance (TEER), fluorescein isothiocyanate–dextran (FITC-dextran) permeability assay, and the localization of Zona Occludens-1 (ZO-1) proteins using immunofluorescence staining and confocal laser scanning microscopy. The results of their experiments indicated that the Th17 cytokines IL-17A, IL-17F, IL-22, and IL-26 might contribute to the development of CRSwNP by promoting a leaky disrupted epithelial barrier as evidenced by a loss of TEER, increased paracellular permeability of FITC-dextrans, and discontinuous ZO-1 immunolocalisation. Intriguingly, neither Th1 nor Th2 cytokines had any bearing on mucosal integrity.[113]

Another domain of ongoing research is investigating the mucociliary response in CRS and the pathways involved. Mucous hypersecretion and ciliary dysfunction are prominent pathophysiological features of CRS; however, the mechanisms causing them are not very well understood.[114,115] Much of the research done nowadays is aimed at providing a better understanding of these phenomena. These types of studies were not possible in the past partly as because of the unavailability of advanced culturing techniques that allows adequate mucociliary differentiation.[116] However, many great advances in knowledge were made possible thanks to ALI and 3D cultures. At the same time, advances in genetics have allowed the identification of multiple mucin genes. Among these, MUC5AC is thought to be one of the major mucin genes in the sinus mucosa.[117] MUC5AC is expressed in increased quantities in CRS.[118]

Several investigators have utilized in vitro models of CRS to identify the stimulants causing MUC5AC overexpression.[119–125] Most recently, Jiao et al. studied the effects of Th1, Th2, and Th17 cytokines on MUC5AC expression and mucociliary function. They exposed the cultured cells to IFN-γ, IL-13, and IL-17, respectively. After exposure, they measured ciliary beat frequency (CBF), and the morphological markers of mucociliary differentiation. CBF was measured using a high-power microscope coupled to a high-speed video camera. The recorded video is then analyzed using a customized software specifically designed to measure CBF. They utilized real-time PCR, Immunofluorescence (IF), Immunocytochemistry (ICC), and Western blotting to explore the resultant mucociliary dysfunction. Their findings suggest that both IFN-γ and IL-13 significantly decreased expression of β-tubulin IV (specific cilia marker), ciliated cell number, and CBF. Additionally, IL-13 caused significant goblet cell hyperplasia and over expression of MUC5AC. Intriguingly, IL-17 appeared to have no effect on ciliary function but did increase the expression of MUC5B, which is another mucin gene.[126] The utility scope of the in vitro models of CRS extends beyond the great insights gained on the pathophysiology of the disease. Therapeutics research is an equally important field of research. In vitro models of CRS could serve as a platform for drug development and testing. Inadequacies in current therapeutic options for CRS necessitates the exploration of novel medications. Moreover, in vitro models have been utilized to study the effects of certain well-known antibiotics and steroids on the intricate CRS pathologic cascade to identify their exact mechanism of action. For instance, Suzuki et al. and Miyanohara et al. investigated the exact mechanism by which low-dose long-term macrolide therapy in CRS works and discovered their immune modulating effects.[77,127] Similarly, Ishinaga et al. explored the mechanism by which glucocorticoids inhibit airway mucus secretion and discovered their modulating effects on MUC genes expression.[128] Indeed, many discoveries are waiting to be uncovered, and many great advances in knowledge are yet to be made. In vitro cell cultures have proven their utility as a valuable tool for CRS research. Three-dimensional (3D) cultures could represent a new avenue leading to more discoveries about CRS and its management. Their full potential is yet to be exploited with further advances in culturing techniques.

CONCLUSION

Animal models of sinusitis have provided great insights into the pathophysiologic processes of CRS. Many animals have been used for this purpose. The rabbit has been the prototypical animal due to the similarity of its sinus anatomy with that of humans. However, its utility is somewhat limited and its use in translational research seems to have plateaued. In recent years, murine models have emerged as a potential alternative. Despite their complex anatomy and difficult handling, they are superior to rabbits in studying sinus disease at both genetic and molecular levels. In vitro models serve to eliminate the translational disparity between animal models and humans producing more reliable and more accurate data. Nevertheless, our review indicates that the conventional monolayer in vitro model is not ideal for a thorough study of sinusitis due to the mechanical effects of the culturing technique on the cultured cells. This may potentially render it less representative of the in vivo process of sinusitis. Culturing techniques have come a long way since the original submerged monolayer cell culture. ALI and three-dimensional cell cultures have the potential for bridging the gap between in vivo and in vitro studies. They may provide further insight into the behavior of human sinonasal epithelial cells in health and in disease and thereby facilitate the development of better treatment strategies than those available at present.

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