

Mouse model of *Aspergillus* and *Alternaria* induced rhinosinusitis

Byung-Hoon Ahn^a, Youn-Ho Park^a, Seung-Heon Shin^{b,*}

^aDepartment of Otolaryngology, School of Medicine, Keimyung University, South Korea

^bDepartment of Otolaryngology, School of Medicine, Daegu Catholic University, Daegu, South Korea

Received 11 March 2008; accepted 11 August 2008

Available online 11 December 2008

Abstract

Objective: Fungi are known to induce the production of chemical mediators from respiratory epithelial cells and have been increasingly recognized as important pathogens in sinusitis. However, the exact role of fungi in the pathogenesis of rhinosinusitis has not been clearly established. This study was performed to improve our understanding of the role of fungi in the pathogenesis of rhinosinusitis by developing an animal model of fungus induced rhinosinusitis.

Methods: Fifty mice (C57BL6/J) were divided into five groups. Sham-operated group was the first group. In the second group, *Aspergillus versicolor* (Group IIa) and *Alternaria alternata* (Group IIb) (10^6 spores/ml) were inoculated into the nasal cavity. In the third group, fungi were inoculated into the nasal cavity in the presence of mucosal scratch (Group IIIa,b) and the fourth group was a nasal mucosal scratch only (Group IV). The fifth was a negative control (Group V). The fungi were inoculated once a week on six occasions and then the animals were sacrificed at 7 weeks. The histological sections were examined in a blind manner for the appearance of neutrophil clusters and epithelial thickness with hematoxylin–eosin stain, and mucus secreting glands using the Alcian blue/periodic acid Schiff stain.

Results: Non-invasive fungal sinusitis had been induced with increased numbers of neutrophil clusters after *Aspergillus* and *Alternaria* exposure. The mice with the mucosal scratch wounds had significantly more inflammatory cell infiltration and epithelial thickening; but eosinophils were not commonly found. The mice with fungal sinusitis had goblet cell hyperplasia and increased mucus secretion in the sinonasal cavity.

Conclusions: Inoculation of fungi in the nasal cavity induced rhinosinusitis in C57BL6/J mice. This mouse model may be used for better understanding of the role of fungi in the pathogenesis of rhinosinusitis.

© 2008 Elsevier Ireland Ltd. All rights reserved.

Keywords: *Alternaria*; *Aspergillus*; Mouse; Rhinosinusitis; Model

1. Introduction

Sinusitis is one of the most frequently reported chronic diseases. In contrast to acute rhinosinusitis, where a bacterial or viral etiology is well established, the etiology of chronic rhinosinusitis (CRS) is not well understood, and its course is often difficult to control, even with aggressive medical and surgical therapies [1,2]. Several studies have attempted to elucidate the mechanisms of CRS and numerous hypotheses have been proposed concerning its pathogenesis, including chronic bacterial infection, inhalant or food allergies, sinus

ostial blockage, and T-cell disturbance caused by aerodynamic factors [3,4].

Paranasal sinus fungal disease was once considered rare. However, the frequency of a diagnosis of fungal sinusitis has increased over the last decade [5,6]. Fungi are being increasingly recognized as important pathogens in cases of sinusitis, but their role in the pathogenesis of sinusitis remains controversial. Fungi are universally present as atmospheric components and reproduced by the formation of spores that are able to enter the respiratory tract by means of inhalation. The pathogenic effects of fungi inoculated into the airway depends upon several factors: (1) the number of spores inhaled, (2) the size of the fungal particles, (3) the integrity of nonspecific and specific host defenses, and (4) the pathobiologic potential or virulence of the fungi [7,8].

* Corresponding author. Tel.: +82 53 650 4528; fax: +82 53 650 4533.
E-mail address: hsseung@cu.ac.kr (S.-H. Shin).

A more specific understanding of sinusitis pathophysiology is needed to improve the management of patients. The development of animal models of sinus disease dates back to the early 20th century; the rabbit has been the most frequently studied animal [9,10]. Their size and accessibility have made rabbit sinus models popular for the investigation of experimental sinusitis. Recently, a mouse rhinosinusitis model has been developed. Although mice do not have frontal and sphenoid sinuses, the anatomy of mouse sinuses are similar to that of humans. The ethmoid and maxillary sinuses are air spaces with connection to the nasal cavity and lined with a pseudostratified ciliated columnar epithelium with some goblet cells [11]. In addition, the mouse has the advantage of genetic manipulation as well as the availability of various reagents for the study of inflammation.

To induce rhinosinusitis in an animal, bacteria, viruses, bacterial toxins, and allergens are used. However, fungi have rarely used in an animal model for rhinosinusitis. The aim of this study was to elaborate a mouse model of fungi induced rhinosinusitis to improve the understanding of the pathogenesis of the development of fungal rhinosinusitis.

2. Materials and methods

2.1. Fungus culture from CRS patients

We obtained 0.2–0.5 ml of nasal secretions from the middle meatus under endoscopic guidance using a sinus aspirator (Xomed, Minneapolis, MN). Nasal secretions were dissolved in 1 ml of normal saline and then a 0.5 ml sample was inoculated on potato dextrose/corn meal agar plates with cyclohexamide. The plates were incubated for 4 weeks at 25 °C. *Alternaria alternata* and *Aspergillus versicolor* were identified morphologically and biochemically using standard methods.

2.2. Study groups and procedure

Fifty, 6-week-old, C57BL6/J female mice weighing 16–22 g were used. They were divided into five groups as follows: Group I: eight sham-operated controls; Group IIa: seven inoculated with *Aspergillus*, Group IIb: eight inoculated with *Alternaria*; Group IIIa: eight inoculated with *Aspergillus* after a nasal mucosal scratch, Group IIIb: seven inoculated with *Alternaria* after a nasal mucosal scratch; Group IV: six with a nasal mucosal scratch only; Group V: six normal controls without an intervention.

To evaluate the influence of mucosal trauma on the pathogenesis of a fungal infection, we studied a mucosal scratch group. The mice were sedated with intraperitoneally administered ketamine hydrochloride (80 mg/kg) and the breathing rate and skin color were evaluated to prevent respiratory failure. After identifying a satisfactory level of anesthesia, a 5-mm midline skin incision over the nasal dorsum was made and the subperiosteal flap was raised

laterally. A 2–3 mm bony hole was carefully drilled with a microdrill over the right nasal cavity. The bleeding was controlled with gauze soaked with a vasoconstrictor. The mucosal scratch wound was produced with a sharp ear cup forceps in a uniform area and size. We attempted to induce rhinosinusitis in the sinonasal cavity by inoculation of 20 µl of normal saline with *Aspergillus versicolor* or *Alternaria alternata* corresponding to 10^7 spores/ml. At each inoculation of fungal solution, the mice were allowed to sniff each drop of solution into their nostrils during normal respiration. The fungal solutions were inoculated every week for 6 weeks and the animals were sacrificed during the seventh week. To exclude the effect of bacterial contamination, isolated fungal spores were stored in 4 °C normal saline with gentamycin for 24 h. The Animal Studies Committee at Daegu Catholic University Hospital approved all protocols.

2.3. Histologic evaluation

The mice were painlessly sacrificed with a lethal dose of intraperitoneally administered pentobarbital sodium (120 mg/kg) during the seventh week. Their decapitated heads were immersed in 10% neutral formalin overnight. The heads were then stripped of the eyes, skin, muscle, and the mandibles were excised. The specimens were decalcified until they were soft in 0.25 mol/l ethylenediaminetetraacetic acid for 24 h. The heads were trimmed with a fresh razor blade, with excision of the anterior portion of the nose and the brain, leaving a portion of the nasal sinus approximately 8 mm in length from anterior to posterior. The resulting blocks were embedded in paraffin and sectioned anterior to posterior at 5-µm thickness.

Three anatomically similar sections were chosen from each mouse for analysis. The first section, the most anterior, was at the level of the maxillary sinuses. The second section, more posterior, was at the end of the maxillary sinuses and the beginning of the complex ethmoid turbinates. The third section, most posterior, contained the brain superiorly.

The appearance of inflammatory cell infiltration, epithelial thickness and epithelial disarray were quantified in the hematoxylin and eosin stained section at 200× and 1000× magnification (Fig. 1). Goblet cell numbers were quantified in periodic acid Schiff (PAS) and Alcian blue (AB) stain at 200× magnification (Fig. 1). All tissue sections were examined blindly with respect to the source of the tissue and the counts were determined at three different mucosal areas for each of the three sections per mouse.

The presence or absence of luminal or submucosal inflammatory cell infiltration were quantified into four categories: 0: no; 1: mild, infiltration scattered but no clumping; 2: moderate, infiltration and cell clumping found in more than two of nasal cavity, maxillary or ethmoid cavity; 3: severe, infiltration and cell clumping at all sinonasal cavity. The epithelial thickness was directly measured on a scale of 1000× magnification and goblet cells were counted at four different areas at 200× magnification.

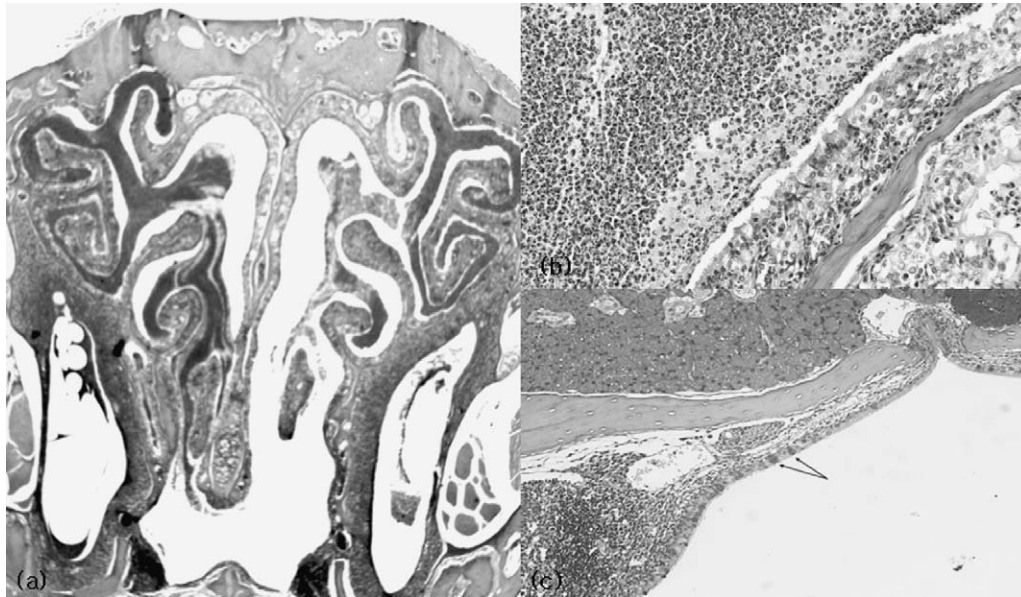


Fig. 1. Example of a nasal-associated lymphoid tissue section of *Alternaria* inoculated mice shows marked inflammatory cell infiltration in the maxillary sinus (arrow) lumen (a, H&E stain; 20 $\times$). In group IIa the infected mice show luminal inflammatory cell infiltration with mucoid discharge (b, H&E stain; 200 $\times$). Periodic acid Schiff and Alcian blue stain shows abundant goblet cells in the maxillary sinus mucosa (c, 200 $\times$).

Within animal comparisons were evaluated with the two-tailed *t*-test and comparison across interventions were evaluated using a one-way ANOVA and the chi-square test. Statistical significance was set under value of 0.05.

3. Results

3.1. Inflammatory change, epithelial disarray

Whereas no or minimal inflammation was noted in the sham operated group, all experimental groups showed a statistically significant increase in the inflammatory cell infiltration of sinonasal cavity. However, the degree of inflammatory change was similar in comparisons between the *Aspergillus* and the *Alternaria*. In addition, no statistically significant difference was identified between the mice with or without a mucosal scratch wound. The histologic examination

of the experimental sinuses revealed luminal and interstitial inflammatory cell infiltrate with mucoid discharge. Most of inflammatory cells were neutrophils, but fungal structures were not observed. Epithelial disarray was more commonly observed in the experimental group than in the sham-operated group and the normal control group (Fig. 2).

3.2. Epithelial thickening

The epithelial thickness was increased at each observed area excluding the olfactory area. The experimental sinuses of the animals with the inoculation of *Aspergillus versicolor*, the group with the scratch wound inoculated with *Aspergillus versicolor* and *A. alternata* showed clear evidence of mucosal edema and statistically significant epithelial thickening. No statistical significance was found in the sham-operated group, the *A. alternata* drop group and the scratch only group (Fig. 3).

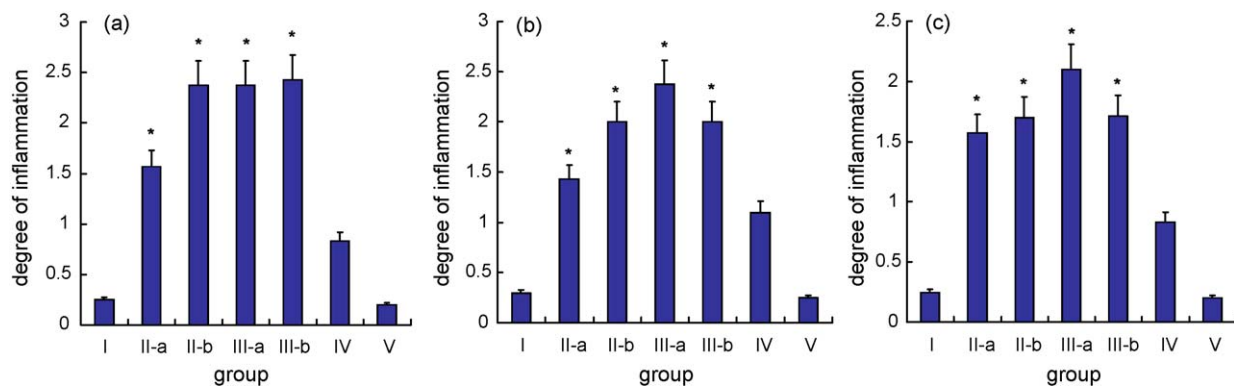


Fig. 2. The degree of inflammatory cell infiltration in the maxillary sinus (a), superior turbinate (b) and nasal septum mucosa (c). Inflammatory cell infiltration was significantly increased in the experimental groups II and III; **p* < 0.05. Degree of inflammatory cell infiltration: 0: no; 1: mild; 2: moderate; 3: severe.

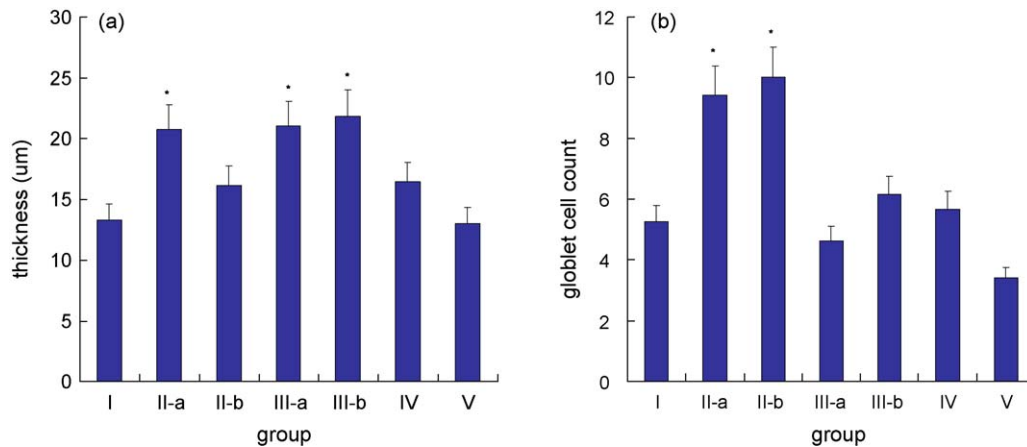


Fig. 3. Within animal comparisons of the mean epithelial thickness (a), and goblet cells. The epithelial thickness was significantly increased in groups IIa, IIIa and IIIb, and the number of goblet cells was increased in groups IIa and IIb; * $p < 0.05$.

3.3. Goblet cell hyperplasia

Increased numbers of goblet cells were observed at the lateral wall of the sinuses and the nasal septum compared to the superior turbinate area. The experimental sinuses of the animals with *Aspergillus versicolor* or *A. alternata* drop only groups demonstrated significantly increased goblet cell numbers by the AB-PAS stain. There were no significant differences in the sham operated group, inoculated fungus group with the scratch and the scratch only group. In addition, we noted marked mucin secretions in the nasal cavity of experimental groups (Fig. 3).

4. Discussion

Fungi are ubiquitous in nature, and the number of fungal species probably exceeds 50,000. However, relatively few species are implicated in human diseases and some of these are commonly associated with airway inflammatory diseases, i.e., *Alternaria*, *Cladosporium*, *Aspergillus*, *Penicillium*, and *Candida*. In general, *Aspergillus* is reported to be the main causative organism of fungal sinusitis but recently *Bipolaris*, *Alternaria*, *Curvularia* and *Exserohilum* have been found to be the major etiologic agents [10,12,13]. In this study, *Aspergillus* and *Alternaria* were used to induce mucosal inflammation. These organisms are known to be common pathogens found in nasal secretion and involve in respiratory tract diseases. Although we could use commercially available lyophilized fungal extract, the fungal components and allergenic potency could be permanently altered, or inactivated by the manipulation. Thus we used fungi cultured from the nasal secretion of human CRS patients.

Th1 cell response of phagocyte-dependent immunity is critical for opposing the infectivity and for clearing pathogenic fungi, whereas Th2 response develops from chronic fungal infection [14]. That is, the degree of cell-mediated immunity directly correlates with the level of

resistance to the fungus, and antibody production inversely correlated with resistance. Although the cause of CRS is unclear, noninfectious inflammation plays a key role in its pathogenesis. This is because of the predominance of eosinophils and mixed mononuclear cells and the relative paucity of neutrophils commonly seen in cases of infectious sinusitis [15,16]. In eosinophilic fungal rhinosinusitis, Inoue et al. [17] hypothesized that the eosinophils not only play a role in the general inflammatory response but also an immunologic defensive role against fungi that are located in mucus. We expected that our mouse model would show increased number of eosinophils. However, in this study the neutrophils were the main inflammatory cell found in the sinus and the nasal cavity. This result might be explained by the different immunologic defense mechanism in the mouse and human against fungi. Since nasal polyps are not found in mouse model that pathophysiology of rhinosinusitis might be different among different species. One other possible reason might be the causative organism of CRS. Although we induced CRS with fungi, fungi just play the role of initiator and bacterial infection follows and plays the main role in the development of CRS that is associated with neutrophil infiltration of tissue. Lindsay et al. [18] made an animal model for chronic eosinophilic rhinosinusitis by sensitizing mice to *Aspergillus fumigatus* extract, and by presenting this antigen to sinonasal mucosa. Their tissue eosinophilia might have been associated with pre-sensitization of the mouse with fungus and our tissue neutrophilia might be associated with fungal inflammatory response in the sinonasal mucosa.

A report on an experimental model of fungal sinusitis in rabbits divided the animals into three experimental groups: *Aspergillus fumigatus* inoculated group, fungi applied after nasal mucosal injury and fungi applied after natural ostial obstruction [19]. Obstruction of the ostium played a major role in the pathogenesis of fungal sinusitis in rabbits. In this study compare with the negative control or sham operated groups, fungi inoculation induced significant increase in the inflammatory cells; however, this increase in the number of

inflammatory cells was not influenced by mucosal scratch wound induced prior to fungi administration. Therefore, our findings suggest that fungi alone can induce mucosal inflammation without mucosal injury in the mouse. The fungi possess several proteins able to produce extracellular protease, which damage mucosal barriers and induce inflammatory responses. The nasal mucosal defense immunity against fungi might be different in the mouse and in rabbits.

The goblet cell hyperplasia caused by the fungi was an interesting finding in this investigation. This finding has been previously described in sinonasal mucosa from a mouse bacterial rhinosinusitis model [20]. Bacteria, fungi and allergens stimulate the release of inflammatory mediators such tumor necrosis factor- α , interleukin-6 (IL-6), IL-8, granulocyte macrophage colony stimulating factor from the sinonasal mucosa. Inflammatory mediators and cytokines induced by infection appear to play a role in the proliferation of goblet cells and the secretion of mucus. However, it is difficult to explain, fungi induced goblet cell hyperplasia without mucosal injury.

In conclusion, intranasal administration of *Aspergillus* and *Alternaria* induced rhinosinusitis in the mouse. The histological evidence of rhinosinusitis revealed inflammatory cell infiltration, epithelial thickening and disarray and goblet cell hyperplasia. This mouse model of fungi induced sinonasal inflammation, may help improve our understanding of the pathogenesis of fungal sinusitis. Future studies should consider presensitization of the mice with ovalbumin or fungi before intranasal administration of fungi.

Acknowledgement

This work was supported by the grant of Research Institute of Medical Science, Catholic University of Daegu (2005).

References

- [1] Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus: induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J Clin Invest* 1995;96:549–57.
- [2] Danese M, Duvoisin B, Agrifoglio A, Cherpillod J, Krayenbuhl M. Influence of naso-sinusal anatomic variants on recurrent, persistent or chronic sinusitis: X-ray computed tomographic evaluation in 112 patients. *J Radiol* 1997;78:651–7.
- [3] Tos M, Sasaki Y, Ohnishi M, Larsen P, Drake-Lee AB. Fireside conference 2. Pathogenesis of nasal polyp. *Rhinol Suppl* 1992;14: 181–5.
- [4] Senior BA, Kennedy DW. Management of sinusitis in the asthmatic patient. *Ann Allergy Asthma Immunol* 1996;77:6–19.
- [5] Cody II DT, Neel III HB, Ferreiro JA, Roberts GD. Allergic fungal sinusitis: the Mayo Clinic experience. *Laryngoscope* 1994;104: 1074–9.
- [6] Ponikau JU, Sherris DA, Kern EB, Homburger HA, Frigas E, Gaffey TA, et al. The diagnosis and incidence of allergic fungal sinusitis. *Mayo Clin Proc* 1999;74:877–84.
- [7] Ferguson BJ, Mitchell TG, Moon R, Camporesi EM, Farmer J. Adjunctive hyperbaric oxygen for treatment of rhinocerebral mucormycosis. *Rev Infect Dis* 1998;10:551–9.
- [8] Fukami M, Norlander T, Stierna P, Westrin KM, Carlsoo B, Nord CE. Mucosal pathology of the nose and sinuses: a study in experimental maxillary sinusitis in rabbits induced by *Streptococcus pneumoniae*, *Bacterioides fragilis*, and *Staphylococcus aureus*. *Am J Rhinol* 1993;7:125–32.
- [9] Bolger WE, Leonard M, Dick Jr EJ, Stierna P. Gram negative sinusitis: a bacteriologic and histologic study in rabbits. *Am J Rhinol* 1997; 11:15–25.
- [10] Adam RD, Paquin ML, Petersen EA, Saubolle MA, Rinaldi MG, Corcoran JG, et al. Phaeohyphomycosis caused by the fungal genera *Bipolaris* and *Exserohilum*. A report of 9 cases and review of the literature. *Medicine (Baltimore)* 1986;65:203–17.
- [11] Bomer K, Brichta A, Baroody F, Boonlayangoor S, Li X, Naclerio RM. A mouse model of acute bacterial rhinosinusitis. *Arch Otolaryngol Head Neck Surg* 1998;124:1227–32.
- [12] Waxman JE, Spector JG, Sale SR, Katzenstein AL. Allergic *Aspergillus* sinusitis: concepts in diagnosis and treatment of a new clinical entity. *Laryngoscope* 1987;97:261–6.
- [13] Manning SC, Schaefer SD, Close LG, Vuitch F. Culture-positive fungal sinusitis. *Arch Otolaryngol Head Neck Surg* 1991;117:174–8.
- [14] Romani L. The T cell response against fungal infection. *Curr Opin Immunol* 1997;9:484–90.
- [15] Drake-Lee AB, Pitcher WR. The clinical and laboratory correlates of nasal polyps in cystic fibrosis. *Int J Pediatr Otolaryngol* 1982;4: 209–14.
- [16] Bernstein JM, Gorfien J, Noble B. Role of allergy in nasal polyposis: a review. *Otolaryngol Head Neck Surg* 1995;113:724–32.
- [17] Inoue Y, Matsuwaki Y, Shin SH, Ponikau JU, Kita H. Nonpathogenic environmental fungi induce activation and degranulation of human eosinophils. *J Immunol* 2005;175:5439–47.
- [18] Lindsay R, Slaughter T, Britton-Webb J, Mog SR, Conran R, Tadros M, et al. Development of a murine model of chronic rhinosinusitis. *Otolaryngol Head Neck Surg* 2006;134:724–30.
- [19] Dufour X, Kauffman-Lacroix C, Goujon JM, Grollier GA, Rodier MH, Klossek JM. Experimental model of fungal sinusitis: a pilot study in rabbits. *Ann Otol Rhinol Laryngol* 2005;114:167–72.
- [20] Jacob A, Faddis BT, Chole RA. Chronic bacterial rhinosinusitis: description of a mouse model. *Arch Otolaryngol Head Neck Surg* 2001;127:657–64.