Persistent mucus accumulation: a consequence of delayed bronchial mucous cell apoptosis in RAO-affected horses?

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This study examined the contribution of delayed apoptosis of bronchial mucous cells to mucus accumulation in equine recurrent airway obstruction (RAO). In pilot studies, Bcl-2, an apoptosis inhibitor, was detected in airway mucous cells of RAO-affected horses in remission and during acute disease, when most mucus was secreted. To study whether delayed apoptosis results in an increase in the number of mucous cells during disease recovery, six RAO-affected and six control horses were fed hay for 5 days to induce inflammation and then pellets for 7 days to partially resolve RAO before euthanasia. RAO-affected horses had more airway obstruction and luminal mucus than control horses under both management systems. At the time of euthanasia, RAO-affected horses had more inflammation and Bcl-2-positive bronchial mucous cells than control animals. In horses with >10 and <10 neutrophils per microliter of bronchoalveolar lavage fluid, >50% and <10% of mucous cells stained positive for Bcl-2, respectively. No differences in mucous cell number or amount of stored mucous substance were observed between RAO-affected and control horses, but in RAO-affected animals, the amount of stored mucous substance decreased as the number of neutrophils in bronchoalveolar lavage fluid increased. Because the number of mucous cells was similar in both groups of horses but only mucous cells of RAO-affected horses expressed Bcl-2 during recovery from acute disease, a conclusive role for Bcl-2 in prolonging bronchial mucous cell life could not be determined. Future studies are needed to compare horses that are kept in remission for prolonged periods when all mucous cells are fully developed.

inflammation; mucous cell metaplasia; recurrent airway obstruction; heaves

RECURRENT AIRWAY OBSTRUCTION (RAO), also known as heaves, is a spontaneous, naturally occurring asthma-like respiratory disease of horses. Acute exacerbations of the disease can be induced by exposure of horses to organic dust, usually from eating hay. RAO is characterized by reversible airway obstruction, which is due to bronchospasm, accumulation of mucoid secretions often containing neutrophils, and remodeling of the airway wall. When horses susceptible to this disease are in remission at pasture, visible accumulations of mucus persist in the trachea, even though inflammation wanes (4, 8). Once these animals return to a dusty environment, the amount of tracheal mucus increases (8).

Accumulation of mucoid secretions in RAO-affected horses is due in part to increased mucus viscoelasticity, with increased expression of the principal mucin gene eqMUC5AC (7, 10). Although mucous cell metaplasia (MCM) and goblet cell hyperplasia are commonly described lesions of RAO, especially in smaller airways (18), the contribution of increased numbers of bronchial mucous cells is unknown.

Bcl-2 is an oncogene on the human chromosome 1 (14; 18) that prolongs cell survival (20, 31), possibly by inhibiting progression of the cell division cycle from the G1 to the S phase, resulting in a 30–60% increase in the length of the G1 phase (19). Ozone, endotoxin, and allergen challenges in rats increase Bcl-2 expression in epithelial mucous cells in the proximal septum of the nose and in the lungs (6, 26–28). A role for Bcl-2 in MCM is suggested by the observation that Bcl-2 expression occurs in ~20–30% of metaplastic mucous cells in epithelium of ozone-challenged rats (25, 27) and that MCM does not resolve as long as Bcl-2 is expressed. Expression of Bcl-2 in spontaneously occurring airway disease has been examined only in cystic fibrosis (15), where Bcl-2 is expressed in mucous cells of cystic fibrosis patients, but not in controls.

This study addresses the hypothesis that delayed apoptosis, as indicated by an increase in Bcl-2-positive mucous cells, contributes, at least in part, to an increase in the number of mucus-secreting cells and airway mucus accumulation in the bronchi of RAO-affected horses. We began with pilot studies on RAO-affected horses in remission and noted Bcl-2 expression in mucous cells of some animals. Next, we examined some RAO-affected animals during acute exacerbation of the disease and observed little stored mucus, so we could not identify mucous cells with certainty. These findings led us to design a study to investigate whether Bcl-2 expression is associated with increased numbers of bronchial mucus-producing cells or may lead to increased mucus synthesis during recovery of horses from the acute stage of the disease. This protocol involved a challenge period followed by a 1-wk reduction in particle exposure. In these animals, we observed Bcl-2 expression in bronchial mucous cells of RAO-affected animals but not controls. However, Bcl-2 expression was not associated with an increase in the number of mucous cells.

MATERIALS AND METHODS

Animals. The RAO-affected animals were from the herd maintained by the Pulmonary Laboratory at Michigan State University and met diagnostic criteria for RAO defined at the International Workshop on Equine Chronic Airway Disease (22). All the horses had previously developed airway obstruction when stabled and fed hay, and the obstruction was reduced by administration of the bronchodilator...
atropine. Horses in the control group had no known history of chronic airway disease and developed no clinical signs of airway obstruction when stabled.

The first pilot study used two control and two RAO-affected adult horses that were euthanized after ≥1 mo at pasture. At the time of euthanasia, the animals showed no signs of respiratory distress. The second pilot study used three RAO-affected and three control horses that had been stabled and fed hay until the RAO-affected animals had visible respiratory distress and wheezed. The main investigation used six control [3 male and 3 female, 11–21 (mean 16.7) yr of age, 399–538 kg body wt] and six RAO-affected [5 male and 1 female, 10–29 (mean 20.8) yr of age, 454–530 kg body wt] adult horses. The Animal Use and Care Committee of Michigan State University approved the studies.

Experimental design. In the pilot investigations, a main axial airway (generation 5) and peripheral airways were sampled. An individual unaware of slide identity quantified the percentages of Bcl-2-positive mucous cells.

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The main study was blinded, with each horse and sampling time point being assigned a random code. The horses were studied in pairs of one RAO-affected and one control animal. After being at pasture for ≥2 (average 8.5) wk, one horse from the pair was stabled, fed hay, and bedded on straw for 5 days. At this point, the horse’s diet and bedding were changed to pellets and shavings, respectively, to reduce the particulate load and severity of airway obstruction in the RAO-affected horses (16). After 7 days on pellets and shavings (day 12), the horse was euthanized. On days 5 and 12, we quantified the severity of airway obstruction by measuring the maximal change in pleural pressure during tidal breathing (ΔPpₘₐₓ), performed bronchoalveolar lavage (BAL), and assigned a tracheal mucus accumulation score. This protocol (Fig. 1) was repeated the following week with the other horse from the pair. The sequence of the pairs, RAO-affected and control, was randomized.

On day 12, the horses were euthanized with pentobarbital sodium (86 mg/kg iv). The thorax was opened, and the lungs were dissected away from other structures, extracted, and grossly examined. From each of eight regions, we collected samples of peripheral parenchyma and an adjacent cartilaginous bronchus (2–6 mm diameter; Fig. 2). The sequence in which the regions were sampled was randomized a priori.

Measurement of ΔPpₘₐₓ. Horses were intubated with an esophageal balloon (Trojan condom, Carter-Wallace) that was sealed over the end of a 240-cm-long polyethylene catheter (3 mm ID, 4.4 mm OD; PE-240, Becton, Dickinson, Franklin Lakes, NJ), with lateral holes drilled in the portion covered by the condom. The catheter was inserted so that the balloon was placed caudal to the base of the heart but cranial to the diaphragm. This location provides a good estimate of the pressure within the pleural cavity at that location (3). Pressure changes within the balloon were detected by a pressure transducer (model DP/45-28, Validyne, Northridge, CA) and recorded on a portable physiograph (Dash model II, Astro-Med, West Warwick, RI). The equipment was calibrated daily against a water manometer. The fluctuations in esophageal pressure were recorded for 20 consecutive breaths. The differences between the peak inspiratory and peak expiratory pressures were calculated to derive ΔPpₘₐₓ.

Tracheal mucus score. The horses were sedated with xylazine hydrochloride (1.1 mg/kg iv) and butorphanol tartrate (0.02 mg/kg iv) before bronchoscopy with a 3-m endoscope (model GIF 300, Olympus America, Melville, MD). The bronchoscope was passed via the nares into the trachea, where the amount of accumulated mucus was scored using a subjective grading system (see Fig. 4) that is reproducible between observers (11).

BAL. The endoscope was wedged in a bronchus. Three 100-ml aliquots of sterile Dulbecco’s phosphate-buffered saline were infused into the lungs and aspirated after each aliquot infusion, and the samples were pooled. Leukocyte density per microliter of BAL fluid (BALF) was assessed with a hemacytometer. To determine the percentage of each type of white blood cell, we counted 200 cells on a cytospin preparation stained with Wright-Giemsa. The percentage of each cell type and the total cell counts were used to calculate the total number of each cell type per milliliter of BALF.

Tissue processing. Parenchyma from each region of the lung was securely clamped using two curved bowel clamps, with tips touching to form two sides of a triangle and the third side serving as the lung margin. One small (2- to 6-mm-diameter) cartilaginous bronchus also was dissected (1 cm long) from each region adjacent to the clamped parenchyma. The bronchus was fixed by immersion in 1% paraformaldehyde-0.1% glutaraldehyde solution (pH 7.4) for 45 min, washed...
three times in fresh 30% ethanol, and stored in the final wash of 30% ethanol at room temperature.

The parenchymal sample was separated from the remaining lung tissue by an incision along the outside of the clamps, which were then removed. The lung “pouch” was perfused with 1% paraformaldehyde-0.1% glutaraldehyde solution (pH 7.4) through a 19-gauge needle inserted into the parenchymal pouch and connected to a system that maintained a constant perfusion pressure of 30 cmH2O. After 15 min, the perfusion apparatus was disconnected, and the lung sample remained for an additional 30 min in a bath of the fixative solution before three ethanol washes and storage in the last wash. Blocks of tissue and bronchi were embedded in paraffin, and 5-µm sections were cut. Two slides were prepared: one was stained with Alcian blue (AB)-periodic acid-Schiff (PAS), and the other was immunohistochemically stained for Bcl-2 and counterstained with AB.

**Immunohistochemistry.** Sections of lung tissue were deparaffinized and rehydrated by routine methods. Antigen retrieval was accomplished by incubation of slides in antigen retrieval solution (Dako, Carpinteria, CA) in a steamer at 98°C for 20 min followed by gradual cooling in the retrieval solution for 20 min at room temperature. Endogenous peroxidase was blocked for 5 min at room temperature with 3% hydrogen peroxide. Endogenous avidin and biotin activities were blocked by incubation of the slides in avidin- and biotin-blocking reagents (Dako) for 10 min each at room temperature. Nonspecific immunoglobulin binding was blocked by incubation of the slides for 10 min at room temperature with a protein-blocking agent before application of the primary antibody. Sections were stained in an autostainer (Dako). The slides were incubated with the primary antibody against Bcl-2 (BD Biosciences/Pharmingen, San Diego, CA) at a dilution of 1:200 for 30 min at room temperature. A streptavidin-immunoperoxidase staining procedure (Dako) was used for immunolabeling (33). The immunoreaction was “visualized” with Nova Red (Vector Laboratories, Burlingame, CA). The sections were counterstained with AB at pH 2.5 and Gill’s 3 hematoxylin. Positive immunohistochemical controls included hyperplastic lymph nodes and lymph nodes with malignant lymphoma from archived equine tissues. For negative controls, the primary antibodies were replaced with TBS buffer.

Sections serial to the airways quantified for mucous cells and Bcl-2 were stained with AB-PAS reagent for stored acidic and neutral mucin components, as described previously (12).

**Morphometric quantification of mucous cell numbers and Bcl-2 expression.** Morphometric analysis of airway mucosa and Bcl-2 positivity was performed using the Scion Image program (Scion, Frederick, MD). The regions of mucosa used for counting mucus-containing cells met the following conditions: 1) the basal lamina was intact, and 2) the section of airway wall was transverse, rather than oblique, so that the apical membrane of mucous cells was aligned with the adjacent surface epithelium. The length of the basal lamina underlying the surface epithelium was measured from a contoured line on the digitized image of the airway epithelium, and the number of Bcl-2-positive and -negative mucous cells per millimeter of basal lamina was counted. The total number of mucous cells was also quantified from AB-PAS-stained tissue to confirm the count.

**Quantification of intraepithelial stored mucosubstances.** Scion Image analysis software was used to adjust pixel density and threshold of intensity to determine the volume density (Vv, ml/mm² basal lamina) of acidic and neutral (AB-PAS-stained) stored mucosubstance along a measured perimeter, as described previously (13, 14).

**Statistical analysis.** Data were examined, and, if they were not normally distributed, logarithmically (log₁₀) transformed. A two-way repeated-measures ANOVA was used to determine the effects of 1) time and disease on ΔPp max, mucus score, and total and differential cell counts, and 2) disease and lung region on the mucous cell counts and their Bcl-2 expression. When significant main effects were detected (P < 0.05), multiple comparisons were made using the Student-Newman-Keuls method. Because of the small number of animals, the significance of the interaction term was set at P < 0.1. Vv in the two groups of horses was compared by Student’s t-test. Statistical analyses were performed by means of a computer software program (SigmaStat, SysStat Software, Point Richmond, CA).

**RESULTS**

RAO horses in remission. AB staining of the tissue sections revealed that only the mucus-containing cells expressed Bcl-2.
and a higher percentage of Bcl-2-positive mucous cells was detected in airway epithelia of RAO-affected horses in remission than in controls (Fig. 3). Only 10–20% of mucous cells of control horses showed Bcl-2 positivity. One RAO-affected horse showed 40–55% of mucous cells with Bcl-2 immunostaining in the bronchial epithelia of the three bronchial generations; the other RAO-affected horse showed Bcl-2 immunostaining only in mucous cells of generation 10 (Fig. 3, C and D). Because these samples were from pilot studies of two horses, statistical significance could not be determined.

**RAO horses during exacerbation.** The epithelium contained few mucus-containing cells in the tissues from control and RAO-affected horses that had been stabled and fed hay to exacerbate disease in the RAO-affected animals. However, many airway epithelial cells from RAO-affected horses immunostained for Bcl-2, whereas those from controls did not (Fig. 3).

**RAO horses in recovery.** \( \Delta P_{\text{pl max}} \) was significantly greater in RAO-affected horses than in controls (Fig. 4A; \( P = 0.045 \)) on days 5 and 12. Despite a decrease in the mean \( \Delta P_{\text{pl max}} \) of RAO-affected animals between days 5 and 12, the change was not significant. In control horses, \( \Delta P_{\text{pl max}} \) remained constant. Mucus scores were significantly higher in the RAO-affected horses (>2.5) than in controls on days 5 and 12 (\( P < 0.001 \); Fig. 4B). There was no effect of time on mucus score in either group. The only statistically significant change in BALF leukocyte numbers was in neutrophils (Fig. 4D): 11.8 ± 5.1 and 216.3 ± 204.7 (SE) neutrophils/µL BALF for control and RAO-affected horses, respectively, on day 5. This difference was not significant, however, because one RAO-affected animal had a low neutrophil count (9 cells/µL). On day 12, the neutrophil count for RAO-affected animals was statistically unchanged from that on day 5 (102.7 ± 48.4). In control horses, the count on day 12 (1.0 ± 0.6) was significantly less than that on day 5 for controls (\( P = 0.007 \)) and on day 12 for RAO-affected animals (\( P < 0.001 \)). The mean counts of macrophages, lymphocytes, mast cells, and eosinophils were 22.5 ± 6.0, 51.1 ± 16.8, 2.5 ± 1.3, and 0.03 ± 0.03 cells/µL, respectively.

In AB-PAS- and Bcl-2-AB-stained tissue, there was no significant disease or regional effect on mucous cell numbers, nor was there a significant difference between groups in \( V_s \) (Fig. 5, A–C). In the RAO-affected horses, \( V_s \) was negatively correlated (\( r = -0.886, P = 0.033 \)) with the logarithmically transformed number of neutrophils in BALF (Fig. 5E).

In every region of the lung, the bronchi of RAO-affected horses contained significantly more Bcl-2-positive mucous cells per millimeter of basal lamina (\( P = 0.011 \)) than controls (Fig. 6, A and B). Because there was no difference in the total number of mucous cells between the two groups, the Bcl-2-positive mucous cells constituted a significantly (\( P = 0.009 \)) greater percentage of mucous cells in RAO-affected animals. Examination of second-generation bronchi and larger bronchioles of each horse demonstrated similar differences in Bcl-2-positive mucous cells between control and RAO horses (Fig. 7, A–F).

In mucous cells of horses with >10 neutrophils/µL BALF (day 12), Bcl-2 expression was observed (Fig. 6C), whereas very little Bcl-2 staining was observed in horses with fewer neutrophils (including 1 RAO-affected animal; Fig. 7, G and H). The same relation was observed when neutrophils were expressed as a percentage of total cells, with the increase in Bcl-2 observed at >20% neutrophils (data not shown). There was no correlation between the number of Bcl-2-positive cells and \( V_s \) or mucus score (data not shown).

**DISCUSSION**

The present study investigated delayed apoptosis as a cause of mucous cell hyperplasia in RAO, an environmentally induced inflammatory obstructive airway disease. Our pilot investigations showed Bcl-2-positive mucous cells in bronchi of RAO-affected animals, but their number was few among the mucous cells of controls that had undergone a similar challenge. Interestingly, in horse airways, Bcl-2 was expressed primarily in mucous cells, as in mucous cells of cystic fibrosis.

**Fig. 4.** A: \( \Delta P_{\text{pl max}} \) in RAO-affected and control horses on days 5 and 12. B: subjective tracheal mucus score in RAO-affected and control horses on days 5 and 12. C: subjective tracheal mucus score on days 5 and 12. D: scoring system is as follows: 0 = no visible mucus, 1 = a single or a few mucus globules, 2 = many mucus globules that are beginning to coalesce, 3 = many mucus globules and a continuous mucus stream, 4 = thick continuous mucus stream and many coalescent mucus globules around the wall of the trachea, 5 = mucus stream filling about one-fourth of the tracheal lumen. D: total neutrophils per microliter of BALF from control and RAO-affected horses on days 5 and 12. Values are means ± SE (\( n = 6 \)). *Significantly different from day 5 within group. †Significantly different from control at the same point.
patients (15). Casalino-Matsuda et al. (2) recently reported that reactive oxygen species induce Bcl-2 expression in human goblet cells. Druilhe et al. (5) found that Bcl-2 is expressed in basal cells of human airways; however, mucous cells were absent in the epithelia. It is possible that Bcl-2 is more abundant in mucous cells and, therefore, is more intensely stained with Bcl-2 antibodies than in basal cells. The significance of these findings needs further investigation.

The continual presence of Bcl-2 immunostaining in the mucous cells of RAO-affected horses after the apparent extrusion of mucus suggests that the mucus was secreted while the Bcl-2 protein remained within the cells. This finding is consistent with the anchoring of Bcl-2 within the membranes of mitochondria or the endoplasmic reticulum (21, 24). These initial findings led us to investigate whether Bcl-2 expression is associated with increased numbers of bronchial mucus-producing cells during the recovery of horses from the acute stage of disease.

The design of the experiment incorporated an organic dust challenge (hay feeding) that is known to induce inflammation and obstruction in RAO-susceptible animals followed by reduction of dust exposure (pellet feeding). One week of exposure to the low-dust environment allowed time for synthesis and storage of mucins and for goblet cell hyperplasia. As expected, the 5-day dust challenge resulted in airway obstruction and accumulation of mucoid secretions in RAO-affected animals: Pmax and tracheal mucus scores were significantly greater in RAO-affected horses than in controls. Although there were functional differences between the two groups of animals, there was no significant difference in the severity of inflammation at the end of the hay challenge (day 5). Others also reported that control animals develop neutrophilic airway inflammation without changes in lung function in response to a similar hay challenge (11, 29). After 1 wk in the lower-dust environment, inflammation had significantly waned in controls, but not in RAO-affected animals, so, at the time of
euthanasia, control animals had significantly fewer neutrophils in BALF than did the RAO-affected horses.

At the time of euthanasia, there were significant differences in the number of Bcl-2-positive mucous cells between the two groups of horses. In RAO-affected animals, Bcl-2-positive cells comprised, on average, >40% of mucous cells, whereas <1% of cells were Bcl-2 positive in controls. Because we were concerned that disease may not be uniform throughout the lung, we examined eight different regions and found similar Bcl-2-positive mucous cells throughout the bronchi and bronchioles. Although Bcl-2-positive cells were virtually absent in all control animals, there was some variation between individual RAO-affected horses. The animal that had very little inflammation on days 5 and 12 was similar to a control animal, in that there were few Bcl-2-positive cells in large and small airways (Figs. 6G and 7H). Figure 6C suggests that Bcl-2-positive cells occurred only in animals in which the neutrophil count was >10 cells/μl (equivalent to 20% neutrophils), and in RAO-affected animals, the number of Bcl-2-positive cells tended to increase with severity of neutrophilic inflammation.

A similar association between the number of Bcl-2-positive mucous cells and numbers of neutrophils in airways also has been reported in rats challenged with two doses of endotoxin (6). However, neutrophils are not essential for Bcl-2 expression, because, in the latter model, neutrophil depletion did not reduce the number of Bcl-2-positive cells.

Although a greater percentage of bronchial mucous cells stained for Bcl-2 in RAO-affected horses with neutrophil inflammation, this was not associated with an increase in the number of mucous cells or the amount of stored mucins (V_s). One possible explanation is that the horses were euthanized before goblet cell hyperplasia, or goblet cell hyperplasia may have occurred in control horses after inflammation. There are no other published reports in scientific journals on the numbers of mucous cells or on the time course of MCM in the bronchi of young healthy horses or even old horses. More investigation of mucous cells in horses, particularly in young animals that have never been exposed to the dust-laden environment of a stable, is needed. If MCM and mucus cell hyperplasia occur within 3–5 days, as in rats (27), the 12-day period of inflammation should have been sufficient to cause goblet cell hyperplasia. Another possibility is that the number of mucous cells was underestimated by AB-PAS staining, because their contents had discharged as a consequence of the neutrophilic inflammation. The absence of stored mucus in the acutely challenged animals and the negative correlation of V_s with neutrophil numbers (r = −0.886, P = 0.033) in the recovering animals lend credence to this possibility.

Another investigation quantified the number of mucous cells in the bronchi of horses with chronic airway disease (32). Horses with spontaneous disease were divided into four groups on the basis of the severity of their lesions throughout the airways. Absolute numbers of goblet cells were not reported; however, in 5-mm-diameter bronchi, the size of the bronchi used in the present investigation, there were no statistically significant differences between groups in the percentage of goblet cells. Interestingly, the percentage of goblet cells was lowest in the most severely affected horses. This observation could be explained by our finding that, in animals with severe inflammation, bronchial mucous cells discharge their contents. For this reason, future studies are needed to determine mucous cell numbers and V_s in RAO-affected horses during a longer period (e.g., 30–60 days) of remission.
If the number of bronchial mucous cells is not increased, what is the role for Bcl-2? It could prevent premature mucous cell death due to the oxidant stress and other factors produced during the inflammatory response. Overexpression of Bcl-2 has been shown to induce accumulation of the active form caspase-9 in the mitochondria, rendering the cells resistant to the redox stress (17). Inasmuch as neutrophils of RAO-affected horses produce reactive oxygen species, Bcl-2 may simply normalize the lifespan of bronchial mucous cells (1).

The presence of Bcl-2 in different airway generations indicates that RAO is a condition that affects the whole tracheobronchial tree and is not limited to the peripheral airways, although the inflammation may be most obvious in that region. Further support for the generalized nature of RAO is provided by the upregulation of eqMUC5AC (10) at all airway levels and the absence of inhibitory nonadrenergic noncholinergic function in large airways (23). Bcl-2-positive mucous cells may synthesize more eqMUC5AC per cell than Bcl-2-negative cells. Therefore, two distinct populations of mucous cells may exist in RAO-affected horses. The Bcl-2-positive mucous cells may represent mucous cells that are capable of synthesizing mucus at a faster rate than the Bcl-2-negative cells. This hypothesis is supported by the fact that, in some RAO-affected horses, ~80% of mucous cells were Bcl-2-positive, suggesting that Bcl-2-positive cells are the first to store mucus following emptying of mucus due to acute inflammation. Increased numbers of Bcl-2-positive cells specialized to synthesize increased mucus would then lead to the higher mucus score in RAO-affected horses than in controls.

This study addressed the role of increased bronchial mucous cell numbers as a potential cause of accumulated amounts of mucoid secretions in the airways of RAO-affected animals. Our data indicate that mucous cell numbers are not increased in bronchi and that stored mucins are released in response to neutrophilic inflammation in RAO-affected horses. Our earlier investigations reported the increased expression of eqMUC5AC, which is necessary to replenish the secreted mucins (10). Furthermore, increased mucus viscoelasticity (7) contributes to delayed mucus clearance (30), further contributing to accumulation of secretions during acute exacerbations of RAO.

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