The Periodontopathic Bacterium *Fusobacterium nucleatum* Induced Proinflammatory Cytokine Production by Human Respiratory Epithelial Cell Lines and in the Lower Respiratory Organs in Mice

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**Key Words**
Chronic periodontitis • *Fusobacterium nucleatum* • Chronic obstructive pulmonary disease • Proinflammatory cytokines

**Abstract**

**Background/Aims:** The most prevalent infectious disease, chronic periodontitis which leads to alveolar bone destruction and subsequent tooth loss, develops due to proinflammatory cytokine production induced by periodontopathic bacteria. Chronic obstructive pulmonary disease (COPD), a non-infectious disease, is the third leading cause of death globally. This condition exacerbates frequently, and which is attributable to proinflammatory cytokine production induced by infection by respiratory microorganisms such as *Streptococcus pneumoniae*. Although a positive association has recently been revealed between chronic periodontitis and COPD, how periodontitis contributes to the pathogenesis of COPD remains unclear. Therefore, we hypothesized that some periodontopathic bacteria are involved in the exacerbation of COPD through the induction of proinflammatory cytokine production by respiratory epithelial cells. In this connection, COPD develops in the airways; however, because most periodontopathic bacteria are anaerobic, they are unlikely to exhibit stable virulence in the lower respiratory organs in humans. Hence, we aimed to elucidate whether...
exposure to heat-inactivated periodontopathic bacteria induces proinflammatory cytokine production by several human respiratory epithelial cell lines and in the lower respiratory organs and serum in mice. **Methods:** Real-time polymerase chain reaction and enzyme-linked immunosorbent assay (ELISA) were used to investigate in vitro induction by heat-inactivated periodontopathic bacteria and *S. pneumoniae* for mRNA expression and protein production of interleukin (IL)-8 and IL-6 by human respiratory epithelial cell lines. ELISA was also used to determine in vivo induction of cytokine production in the lower respiratory organs and serum of intratracheally heat-inactivated *Fusobacterium nucleatum*-inoculated mice. **Results:** Some, but not all, periodontopathic bacteria, especially *F. nucleatum*, strongly induced IL-8 and IL-6 production by BEAS-2B bronchial epithelial cells. In addition, *F. nucleatum* induced IL-8 production by A549 alveolar epithelial cells as well as IL-8 and IL-6 production by Detroit 562 pharyngeal epithelial cells. Furthermore, *F. nucleatum* induced considerably higher cytokine production than *S. pneumoniae*. This was also observed in the entire lower respiratory organs and serum in mice. **Conclusion:** Exposure to increased number of *F. nucleatum* potentially induces proinflammatory cytokine production by human bronchial and pharyngeal epithelial cells, which may trigger exacerbation of COPD.

**Introduction**

Periodontitis, one of the most prevalent diseases worldwide, is a polymicrobial infection and multifactorial disease, but it is simply characterized by chronic inflammation in the periodontium [1, 2]. If left untreated, this condition leads to alveolar bone destruction and subsequently results in tooth loss, during which major periodontopathic bacteria, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, are known to be involved in the induction of proinflammatory cytokine production [1, 2]. Moreover, it may result in systemic complications, such as pre-term birth, diabetes, and cardiovascular diseases [1, 2]. Additionally, we have previously reported that *P. gingivalis* and *F. nucleatum* induced the reactivation of latent viruses, namely human immunodeficiency virus and Epstein–Barr virus [3, 4].

Chronic obstructive pulmonary disease (COPD) is an obstructive respiratory disease characterized by chronic inflammation in the lower respiratory tract and emphysema, which is classified as a non-infectious disease with a global prevalence of approximately 10% among individuals aged >40 years [5]. It is also characterized by progression in relation to the repetition of its exacerbation [5]. Therefore, COPD exacerbations have been of major importance because of their prolonged detrimental effects on patients and the resulting high healthcare costs. These exacerbations are represented by increased number of inflammatory cells including neutrophils, elevated levels of proinflammatory cytokines including chemokines, and higher levels of proteases in the respiratory tract [5, 6]. Among host–microbe interactions, a cross-relationship between COPD exacerbations and infection by respiratory bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, has been extensively delineated [6, 7]. These bacteria have been detected in clinical specimens, such as bronchoalveolar lavage fluid (BALF) and sputum, obtained from patients with COPD [6, 7]. They induce the production of several proinflammatory cytokines by respiratory epithelial cells, including interleukin (IL)-8 and IL-6, which in turn attracts neutrophils and mediates their degranulation [5-7]. Thus, these respiratory bacteria amplify inflammation in respiratory epithelial cells and activate macrophages, resulting in the subsequent destruction of normal tissues. In this regard, these cytokines detected in the BALF, sputum, or exhaled breath condensates of patients with COPD are also responsible for COPD exacerbation owing to their proinflammatory nature [6, 8-11].

Evidence accumulated over the past decade indicates that chronic periodontitis aggravated by poor oral hygiene is a risk factor for COPD [12-15]. A recent meta-analysis of 14 studies involving 3988 patients with COPD has revealed a significant association between chronic periodontitis and COPD [12]. For example, alveolar bone loss, a typical symptom
of chronic periodontitis, is associated with an increased risk of COPD [14, 15]. In addition, periodontopathic bacteria are frequently detected in clinical specimens from patients with COPD [16-19]. Furthermore, periodontal interventions significantly reduce the frequency of COPD exacerbations [20, 21]. Additionally, on an average, an individual generates and ingests up to 1.5 l of saliva per day, and saliva contains an enormous number of bacteria released from the mouth [22]. Approximately half of all healthy adults experience salivary aspiration during sleep [23]. Further, in patients with COPD, the risk of aspiration has increased because these patients have reduced laryngopharyngeal sensitivity [24]. Thus, these observations suggest that periodontopathic bacteria present in saliva are aspirated through the pharynx into the lower respiratory tract, thereby triggering COPD exacerbations. However, how periodontopathic bacteria contribute to COPD exacerbations remains unexamined.

As the majority of periodontopathic bacteria are anaerobic, they are unlikely to exhibit stable virulence in the respiratory tract. In addition, it is unclear how long these bacteria survive in vitro and in vivo. Therefore, we examined whether some periodontopathic bacteria induced proinflammatory cytokine production by several human respiratory epithelial cells merely on exposure to heat-inactivated bacteria.

In the present study, for the first time, we present a causal association between periodontopathic bacteria, especially F. nucleatum, and the inducible production of proinflammatory cytokines, namely IL-8 and IL-6, both of which are surrogate clinical mediators of COPD exacerbations, from human bronchial and pharyngeal epithelial cells and in the lower respiratory organs and serum in mice.

Materials and Methods

Cell culture

Human bronchial (BEAS-2B), pharyngeal (Detroit 562), and alveolar (A549) epithelial cells were purchased from ATCC (Manassas, VA, USA) and maintained at 37°C in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc., Rockford, IL, USA), penicillin (100 U/ml), and streptomycin (100 mg/ml) as described previously.

Bacterial culture and sample adjustment

F. nucleatum ATCC 25586, P. gingivalis ATCC 33277, Prevotella intermedia ATCC 25611, Aggregatibacter actinomycetemcomitans Y4, Tannerella forsythia JCM 10827, Treponema denticola ATCC 35405, S. mitis JCM 12971, S. salivarius JCM 5707, and S. pneumoniae ATCC 49619 were used throughout this study. S. mitis, S. salivarius, and S. pneumoniae were cultured in brain heart infusion broth (BHB; Becton, Dickinson and Company, Sparks, MD, USA). F. nucleatum, P. gingivalis, P. intermedia, and A. actinomycetemcomitans were cultured in BHB supplemented with 5 μg/ml hemin and 0.5 μg/ml menadione. T. forsythia was cultured in BHB supplemented with 5 μg/ml hemin, 0.5 μg/ml menadione, 10 μg/ml N-acetylmuramic acid, and 5% fetal calf serum. T. denticola was cultured in new oral spirochete broth as previously described [4, 25].

All cultures were incubated at 37°C for 24–72 h and grown in an anaerobic chamber (Te-Her Anaerobox, Hirasawa Co. Ltd., Tokyo, Japan) under an aerobic condition of 10% H₂, 10% CO₂ and 80% N₂. Subsequently, culture supernatants were obtained via centrifugation at 7000 × g for 10 min at 4°C and then filtrated through a 0.22-μm pore filter membrane. In parallel, the bacterial cell density was adjusted to 1.0 × 10⁹ CFU/ml, and the bacterial suspension was heat-inactivated at 60°C for 1 h and then stored at −80°C until use.

mRNA preparation and real-time polymerase chain reaction (PCR)

The experimental procedures for RNA purification and real-time PCR were performed as previously described [26]. Briefly, BEAS-2B cells were washed once with ice-cold phosphate-buffered saline (PBS) and homogenized using a QIAshredder (QIAGEN, Alameda, CA, USA), while total RNA was purified using an RNeasy Mini Kit (QIAGEN). For cDNA synthesis, total RNA (1 μg) was reverse transcribed using an RNA PCR kit (PrimeScript; Takara Bio, Shiga, Japan). The resulting cDNA mixture was subjected to real-time PCR
analysis using SYBR Premix Ex Taq solution (Takara Bio) containing 5 μM sense and antisense primers. The primer sequences used for the amplification of each gene were as follows: IL-8, forward (5-CTT GTC ATT GCC AGC TGT GT-3) and reverse (5-TGA CTG TGG AGT TTT GGC TG-3); IL-6, forward (5-TTC GGT CCA GTT GCC TTC TC-3) and reverse (5-GAG GTG AGT GGC TGT CTG TG-3); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward (5-ACC AGC CCC AGC AAG AGC ACA AG-3) and reverse (5-TTC AAG GGG TCT ACA TGG CAA CTG-3). PCR assays were performed using a TP-800 Thermal Cycler Dice Real-Time System (Takara Bio) and analyzed using the software provided by the device manufacturer. The thermal cycling conditions were 40 cycles at 95°C for 5 s, 60°C for 30 s, and 72°C for 1 min. All real-time PCR experiments were performed in triplicates, and the specificity of each product was verified via a melting curve analysis. The calculated gene expression levels were normalized to GAPDH mRNA levels.

Cytokine measurements

The IL-8 and IL-6 concentrations in the cell culture supernatants were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the procedures recommended by the manufacturer. All experiments were performed in triplicate and data are presented as the mean ± standard deviation.

Animals and inoculation procedures

All animal experiments were conducted in accordance with the Regulations and Guidelines on Scientific and Ethical Care and Use of Laboratory Animals of the Science Council of Japan, enforced on June 1, 2006. The study protocol was approved by the Institutional Animal Care and Committee of Nihon University School of Dentistry (Permit Number: AP16D047). Specific pathogen-free male C57BL/6Jc mice aged 7 weeks were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed under standard conditions within the animal care facility at the Nihon University School of Dentistry, Tokyo, Japan. All mice were anesthetized with aspirating isoflurane and inoculated with bacteria intratracheally. The final bacterial loads of F. nucleatum and S. pneumoniae were 1 × 10^8 CFU/mouse suspended in a total volume of 50 µl of PBS. PBS-treated mice (control) and bacteria-treated mice were housed in separate cages for 2 h. The Nihon University of Animal Care and Use Committee approved the protocol.

Sample collection from mice and cytokine quantitation

Mice were sacrificed after a 2-h exposure to bacteria. Then, whole blood was collected from the abdominal aorta, and the trachea and lungs were surgically removed. Serum was separated by centrifuging blood samples and stored at −80°C until use. Tracheal and lung tissues were homogenized in radioimmunoprecipitation assay buffer using a BioMasher (Nippi, Tokyo, Japan) and centrifuged. The supernatants were collected and stored at −80°C until use. Production of the inflammatory cytokines KC and IL-6 in the serum and tissue homogenates was determined using ELISA kits (R&D Systems). The level of each cytokine was normalized to the amount of total protein.

Results

Effects of human oral bacteria on proinflammatory cytokine production by a human bronchial epithelial cell line

Among more than 700 species of human oral bacteria, a few gram-negative anaerobes have been linked to human chronic periodontitis. First, we investigated the effects of human oral bacteria on IL-8 and IL-6 production by BEAS-2B human bronchial epithelial cells. BEAS-2B cells were incubated for 12 h with and without the heat-inactivated bacterial suspension, and the protein levels of both cytokines secreted into the supernatants were then measured using ELISA. As shown in Fig. 1, human periodontopathic bacteria, such as P. gingivalis, F. nucleatum, T. forsythia, P. intermedia, and A. actinomycetemcomitans, substantially induced IL-8 and IL-6 production by BEAS-2B cells. In particular, F. nucleatum induced the release of extremely high concentrations of both cytokines. Conversely, T. denticola negligibly induced cytokine production. In parallel, two oral streptococci predominant in the human saliva, namely S. salivarius and S. mitis, failed to elicit cytokine production.
**F. nucleatum induced mRNA expression and protein production of IL-8 and IL-6 by human bronchial epithelial cell line**

As only *F. nucleatum* induced extremely high proinflammatory cytokine production among heat-inactivated periodontopathic bacteria and as it is predominant in periodontitis lesions and is found in clinical specimens obtained from patients with COPD [17-19], real-time PCR analysis was performed to investigate the effects of *F. nucleatum* on the mRNA expression of these cytokines in BEAS-2B cells. As shown in Fig. 2a, *F. nucleatum* markedly upregulated IL-8 and IL-6 mRNA levels after a 1-h exposure, with peaking after a 24-h exposure (636 ± 172- and 217 ± 35-fold, respectively). Fig. 2b demonstrates that *F. nucleatum* also markedly induced IL-8 and IL-6 production between 1- and 24-h exposures. To clarify the cause-and-effect relationship between the number of bacteria and the cytokine expression level, we further examined density dependency using *F. nucleatum* at densities between $1 \times 10^6$ and $1 \times 10^8$ CFU/ml. As shown in Fig. 2c and d, *F. nucleatum* elicited mRNA expression and production of both cytokines in a density-dependent manner.

**Effects of F. nucleatum on IL-8 and IL-6 production by human respiratory epithelial cell lines**

Next, we examined whether heat-inactivated *F. nucleatum* could induce proinflammatory cytokine production in other human respiratory epithelial cell lines, such as A549 alveolar cells and Detroit 562 pharyngeal cells. As shown in Fig. 3a, IL-8 production was induced in A549 cells, whereas IL-6 production was not. Meanwhile, *F. nucleatum* induced the production of both cytokines by Detroit 562 cells in a density-dependent manner, as shown in Fig. 3b.

**Fig. 1. Effects of human oral bacteria on IL-8 and IL-6 production by human bronchial epithelial cells.**

BEAS-2B cells were treated with or without indicated heat-inactivated oral bacteria at a bacterial density equivalent to $1 \times 10^6$ CFU/ml for 12 h, after which IL-8 and IL-6 protein levels in the cell culture supernatants were determined using ELISA and expressed as pg/ml. Data are presented as the mean ± SD.
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*F. nucleatum* induced IL-8 and IL-6 production by human respiratory epithelial cell lines more strongly than *S. pneumoniae*

Because *S. pneumoniae* contributes to COPD exacerbations by inducing the production of proinflammatory cytokines, including IL-8 and IL-6 [5, 7], we compared this induction by *S. pneumoniae* and *F. nucleatum* in three human respiratory epithelial cell lines. As shown in Fig. 4, *S. pneumoniae* induced IL-8 production by BEAS-2B, A549, and Detroit 562 cells, albeit at much lower levels than *F. nucleatum*. A similar induction was also observed for IL-6 production by BEAS-2B and Detroit 562 cells.
Fig. 3. Effects of *Fusobacterium nucleatum* on IL-8 and IL-6 production by two respiratory epithelial cell lines. Human alveolar (A549) (a) and pharyngeal (Detroit 562) (b) epithelial cells were incubated with heat-inactivated *F. nucleatum* at 1 × 10⁷ CFU/ml for 12 h. IL-8 and IL-6 protein levels in the cell culture supernatants were determined using ELISA and expressed as pg/ml. Data are presented as the mean ± SD. *; a value below the detection limit.

**Effects of *F. nucleatum* and *S. pneumoniae* on KC and IL-6 production in mice**

To compare the effects of *F. nucleatum* and *S. pneumoniae* on *in vivo* cytokine production, we further investigated the production of KC, an IL-8 homologous neutrophil chemoattractant in mice, as well as that of IL-6 in the presence of heat-inactivated *F. nucleatum* or live *S. pneumoniae*. This experiment was conducted within a short period such as 2 h, during which bacterial infection was not established [27] in the lower respiratory organs in mice. As shown in Fig. 5a, both heat-inactivated and live *S. pneumoniae* appeared to induce KC and IL-6 production in the lungs of mice. However, there were no statistical differences in terms of KC and IL-6 production between the bacteria-treated and control mice. Conversely, *F. nucleatum* alone induced the prodigious production of both cytokines, and this finding was consistent with our *in vitro* results. Remarkably, as shown in Fig. 5b, although the mouse trachea is as short as 1 cm in length, this tendency was also observed in such a shorter respiratory
Fig. 4. Comparison of the effects of *Fusobacterium nucleatum* and *Streptococcus pneumoniae* on inflammatory cytokine production. BEAS-2B (a), A549 (b), and Detroit 562 (c) cells were treated with heat-inactivated *F. nucleatum* (1 × 10⁸ CFU/ml) or heat-inactivated *S. pneumoniae* (1 × 10⁸ CFU/ml) for 12 h. IL-8 and IL-6 levels in the cell culture supernatants were determined using ELISA and expressed as pg/ml. Data are presented as the mean ± SD. *; a value below the detection limit.
Fusobacterium nucleatum and Streptococcus pneumoniae on the production of proinflammatory cytokines in vivo. Mice (n = 6) were treated with heat-inactivated F. nucleatum (1 × 10^8 CFU/mouse), heat-inactivated S. pneumoniae (1 × 10^8 CFU/mouse), or live S. pneumoniae (1 × 10^8 CFU/mouse) for 2 h. KC and IL-6 protein levels in the lungs (a), trachea (b), and serum (c) were determined using ELISA and expressed as pg/mg protein or pg/ml. Data are presented as the mean ± SD.

Discussion

As bacteria in the saliva can be aspirated into the lower respiratory tract through the pharynx, many studies have described a positive association between chronic periodontitis and COPD [12-15, 20, 21]. For example, major periodontopathic bacteria, such as F. nucleatum and P. gingivalis, have been isolated from clinical samples of patients with COPD [16-19]. However, the mechanism by which periodontopathic bacteria are involved in the
pathogenesis of COPD has not been delineated. In contrast, respiratory bacteria such as *S. pneumoniae* induce proinflammatory cytokine production by lower respiratory epithelial cells, which plays a key role in initiating and orchestrating inflammations in patients with COPD by recruiting and activating multiple inflammatory cell types in the lower respiratory tract [5-7]. In this regard, major periodontopathic bacteria also appear to be proinflammatory [1]. Based on these reports, we hypothesize that exposure to increased number of periodontopathic bacteria during aspiration as a result of poor oral hygiene potentially exerts proinflammatory effects on the respiratory epithelium in patients with COPD. However, it is unclear how long these bacteria survive when incubated with human respiratory cells under experimental conditions. Moreover, we were rather interested in inducible effects associated with heat-inactivated periodontopathic bacteria on proinflammatory cytokine production by human respiratory epithelial cells. In addition, surface structures and some biological functions of gram-negative bacteria inactivated by heat are preserved compared with those of live bacteria [28, 29]. Therefore, we used heat-inactivated bacteria in the present study. We first found that some periodontopathic bacteria, but not all bacteria, function as stimulants to induce IL-8 and IL-6 production by the human bronchial epithelial cell line. Next, we found that among the periodontal bacteria, *F. nucleatum* alone intensely induced cytokine production by several human respiratory epithelial cell lines, including bronchial and pharyngeal epithelial cells, in a density-dependent manner. Further, *F. nucleatum* strongly induced cytokine production in the lower respiratory organs in mice. These observations suggest that *F. nucleatum*, even when devoid of its vitality, is a potent proinflammatory stimulant for the human bronchial and pharyngeal epithelial cells and respiratory organs in mice.

IL-8, a potent neutrophil chemoattractant and activator, has been associated with the pathogenesis of COPD, ascribable to the accumulation and degranulation of neutrophils, which are responsible for the subsequent destruction of normal tissues [7]. Many studies have demonstrated that IL-8 levels are markedly increased in the BALF or induced sputum of patients with COPD and that this induction is correlated with the increased proportion of neutrophils as well as the increased concentration of elastase [7-9, 30]. There is also a significant correlation between IL-8 levels and bacterial infection [9, 31]. Moreover, cultured respiratory epithelial cells from patients with COPD secrete more IL-8 than that secreted by those from normal smokers, indicating an amplified response [32]. Likewise, IL-6 activity is involved in the stimulation of acute-phase protein synthesis, leukocyte recruitment, B-cell differentiation, and T-cell activation in many chronic inflammatory diseases [7]. The IL-6 concentration is also increased in the BALF, induced sputum, and exhaled breath condensate of patients with COPD, particularly during its exacerbations [7, 10, 11, 33]. In addition, IL-6 levels increase in the plasma during COPD exacerbations [34]. Our interspecies comparison of cytokine production suggested that *F. nucleatum* is a more potent proinflammatory stimulant in the lower and pharyngeal epithelia of humans than *S. pneumoniae*. Moreover, proinflammatory cytokines are multifunctional and exert their influences in paracrine and autocrine manners on modulating inflammatory responses of respiratory epithelial cells [5-7]. Thus, our findings suggest that the inflammation that triggers COPD exacerbation is caused by exposure to *F. nucleatum* and by the absorption of paracrine proinflammatory cytokines released from the pharyngeal epithelium, which is induced by a precedent exposure to *F. nucleatum*. In fact, periodontopathic bacteria are present in high numbers in periodontitis lesions, tongue dorsum, and saliva [22, 35]. In addition, in patients with COPD, the risk of aspiration has increased because these patients have reduced laryngopharyngeal sensitivity and an increased risk of mild cognitive impairment [24, 36], resulting in swallowing reflex impairment [37]. These observations along with our findings may support our hypothesis that an increased number of periodontopathic bacteria in the saliva as a result of poor oral hygiene increases the risk of COPD exacerbation.

Respiratory epithelial cells represent the first line of defense against pathogens that colonize the respiratory tract. In addition, bacteria adhere to respiratory epithelial cells via specific adhesin–receptor interactions. Recognition of bacteria by respiratory epithelial
cells possibly induces the production of proinflammatory cytokines. Similar to other infectious diseases, adhesion to and subsequent invasion of the gingival sulcular epithelium by periodontopathic bacteria are critical steps in the initiation of chronic periodontitis. However, because heat-inactivated bacteria were used to stimulate respiratory epithelial cell lines in the present study, our findings indicate that infection was not necessarily required for the observed induction of proinflammatory cytokine production. In fact, heat-inactivated periodontopathic bacteria, such as *F. nucleatum* and *P. gingivalis*, can modulate the expression of proinflammatory cytokines from several types of human cells, such as macrophages, peripheral blood mononuclear cells, and gingival epithelium [38-40]. Additionally, in the lungs and trachea of intratracheally inoculated mice, we found that significantly greater proinflammatory cytokine production was induced by heat-inactivated *F. nucleatum* than by heat-inactivated or live *S. pneumoniae* during 2-h incubation, a period during which engulfed live bacteria were not expected to multiply [27]. Interestingly, the observed elevation of proinflammatory cytokines in the serum of *F. nucleatum*-treated mice suggested that these cytokines released from the lower respiratory epithelial cells reached the circulatory system within a short period.

Interactions between the surface molecules of *F. nucleatum* possibly involved in the observed cytokine production in this study, expression of pattern recognition receptors such as Toll-like receptors (TLRs) on the respiratory epithelial cell membrane, and expression of lipopolysaccharide on the outer membrane of *F. nucleatum* are important for the first step of proinflammatory cytokine production [41].

Till date, we have observed that TLR2 is involved in the observed cytokine production (data not shown). Further studies are, therefore, required to identify which bacterial structures and host epithelial receptors are possibly involved in these events. However, our findings suggest that periodontopathic bacteria potentially exacerbate COPD by proinflammatory cytokine production and provide a scientific basis to support the importance of maintaining good oral hygiene.

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**Disclosure Statement**

The authors have no conflicts of interest to declare.

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