

Porphyromonas gingivalis induced inflammatory responses and promoted apoptosis in lung epithelial cells infected with H1N1 via the Bcl-2/Bax/Caspase-3 signaling pathway

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Abstract. The aim of the present study was to investigate the effects of *Porphyromonas gingivalis* (*P. gingivalis*) on inflammatory cytokine and nitric oxide (NO) production in lung epithelial cells infected with H1N1, and the underlying mechanisms. Lung epithelial cells were co-infected with *P. gingivalis* and H1N1. The concentrations of tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6 were detected via an ELISA, and the concentration of NO was detected by the nitrate reductive enzymatic method at 4, 8, 12 and 24 h following infection. The expression levels of inducible NO synthase (iNOS) was detected by western blotting. The apoptotic rate of lung epithelial cells was detected by flow cytometry. The relative protein expression levels of B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax) and caspase-3 in lung epithelial cells were detected by western blotting. Compared with the control group, the concentration of the inflammatory cytokines TNF- α , IL-1 β and IL-6 exhibited a significant increase ($P < 0.05$) in the viral-infected, bacterial-infected and co-infected groups. The concentration of NO also increased significantly ($P < 0.05$), along with the rise in the expression levels of iNOS ($P < 0.05$) and the increase in

the apoptosis rate of lung epithelial cells ($P < 0.05$). The relative expression levels of caspase-3 and Bax proteins were increased significantly in the viral- and bacterial-infected groups when compared with the control. The relative expression levels of Bcl-2 protein exhibited a significant decrease in lung epithelial cells following the co-infection with *P. gingivalis* and H1N1 compared with the control ($P < 0.05$). The results of the present study revealed that the combination of *P. gingivalis* and H1N1 infection in lung epithelial cells may promote the production of inflammatory cytokines and increase NO production, leading to increased levels of apoptosis in lung epithelial cells via the Bcl-2/Bax/caspase-3 signaling pathway.

Introduction

Periodontal disease is a chronic bacterial condition associated with tooth loss. Periodontitis remains a major cause of tooth loss in adults worldwide: The World Health Organization recently reported that severe periodontitis exists in 5-20% of adult populations (1). In recent years, the association between periodontal disease and systemic health has acquired a high level of attention. As medical research has developed, it has been reported that periodontal disease may involve the local oral periodontal tissue, and it may also be associated with cardiovascular disease (2), diabetes (3), rheumatoid arthritis (4), respiratory tract infection (5) and other systemic diseases.

Porphyromonas gingivalis (*P. gingivalis*) is the main pathogenic bacteria involved in periodontal disease (6). Plaque formation is the initial factor of periodontal disease; saliva-derived membranes, which may be colonized by bacteria, are formed at the beginning of production (7). With the progression of periodontal disease, the pathogenic bacteria in the oral cavity and oropharynx increases constantly, resulting in various types of pneumonia and respiratory infections as they arrive in the lower respiratory tract and lungs (8). Pneumonia-associated pathogens may be detected in the dental plaque of patients with pneumonia, and periodontal pathogens may be cultured from lung lavage fluid (9). Previous

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clinical studies (1,10) have indicated that periodontal infection may significantly increase the incidence of fatal pneumonia in patients on a ventilator, and there is an increased risk of mortality from respiratory diseases, such as pneumonia, in elderly patients with periodontitis. A therapeutic studies has revealed that the incidence of respiratory infection decreased in elderly patients with periodontitis who received regular oral treatment for periodontal disease (11). In addition, previous investigation has provided information on the formation and maintenance of good oral health (11). Epidemiological surveys demonstrated that the risk of respiratory disease is increased due to mouth-breathing habits and periodontal disease, which may be due to the airflow via the oral cavity, the lower respiratory tract and the lungs, thereby introducing periodontal pathogens into the lungs where an infection may occur (12,13).

In the process of periodontal epithelial tissue and bone tissue destruction, *P. gingivalis* serves an important role; it secretes a large number of toxic factors to stimulate the host cells into producing the inflammatory cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor- α (TNF- α), which lead to physiological and pathological damage (14). Influenza A virus (IAV) H1N1 is a lethal pathogen that infects humans and animals. It is globally pervasive and is associated with high rates of morbidity and mortality (15). Normally, the number of macrophages in the alveolar cavity is low; a variety of inflammatory cytokines, including ILs, TNFs, chemokines, cytokines and nitric oxide (NO), are produced when lung epithelial cells are severely infected, which is closely associated with lung injury (16). A study on the modeling of bacterial or viral infection in mice demonstrated that IAV H1N1 may increase the risk of mice becoming infected with pneumococcus (17). Additionally, higher expression levels of inflammatory cytokines, monocyte chemoattractant protein 1, chemokines and granulocyte colony stimulating factor were observed.

In the present study, lung epithelial cell infection, co-infection of *P. gingivalis* and IAV H1N1, and the expression levels of inflammatory cytokines and NO were investigated in order to study the effects of *P. gingivalis* on the inflammatory cytokine and NO production within lung epithelial cells infected with H1N1.

Materials and methods

Cells and viruses. BEAS-2B cells were acquired from the Department of Immunology and Microbiology of Jinzhou Medical University (Jinzhou, China), and were grown in RPMI-1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd., Hangzhou, China). All cells were maintained under the recommended culture conditions and incubated at 37°C in a humidified environment with 5% CO₂. *P. gingivalis* was acquired from the Department of Oral Biology of Jinzhou Medical University and routinely grown in Brain Heart Infusion (BHI) blood agar medium or BHI broth (Beijing Aoboxing Biotechnology Co., Ltd., Beijing, China), supplemented with 0.5% yeast extract, hemin (10 µg/ml) and vitamin K (1 µg/ml). Following incubation at 37°C for 4 days, the bacterial number in the culture medium was determined by reading the optical density values at 600 nm using a spectrophotometer, and comparing them

against a curve derived from a standard plate count. The influenza PR/8/34 (H1N1) virus employed in the present study was obtained from the Department of Immunology and Microbiology of Jinzhou Medical University. The virus was grown in the chorioallantoic fluid of 10-day-old embryonic hen eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) at 37°C for 2 days. Following harvesting, the allantoic fluid was filtered with a 0.22-µm cellulose acetate membrane. The filtered liquid was stored in small aliquots at -70°C until further use. The BEAS-2B cells were randomly and equally divided into four groups and treated under the following conditions at 37°C with 5% CO₂: control group (no bacterial and viral infections) H1N1 virus-treated [multiplicity of infection (MOI)=2:1]; *P. gingivalis*-treated and H1N1 virus plus *P. gingivalis*-treated (mixed infection) groups. Briefly, cells were plated in a 6-well plate at a density of 2x10⁶ cells/well and adhered for 24 h at 37°C in a 5% CO₂ incubator. Bacteria and viruses were suspended in 2 ml serum and antibiotic-free RPMI-1640 medium. The *P. gingivalis* infection group and mixed infection group cells were infected with *P. gingivalis* (MOI=100:1) and were cultured at 37°C with 5% CO₂ for 2 h. Then, bacterial infection liquid was removed and H1N1 virus (MOI=2:1) was used to infect the virus and mixed infection group at 37°C with 5% CO₂ for 1 h. Maintenance medium (2 ml; RPMI-1640 with 0.5% FBS) was subsequently added to each well. Untreated cells were used as a negative control group. All groups were recorded as 0 h at this time.

ELISA for TNF- α , IL-1 β and IL-6. At 4, 8, 12 and 24 h post-infection, the cell culture media of all groups were collected and centrifuged at 800 x g for 20 min at 4°C to remove debris. The supernatants were collected (400 µl) to measure the concentrations of TNF- α , IL-1 β and IL-6 with an ELISA-based capture assay, using commercial TNF- α (cat. no. PT512), IL-1 β (cat. no. P1301) and IL-6 (cat. no. P1326) ELISA kits (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instruction. For all cytokines, the optical density was determined within 15 min upon the addition of 50 µl Stop Solution (2 N sulfuric acid), using a microplate reader (ChroMate® 4300, Awareness Technology Inc., Palm City, Florida, USA) set to 450 nm, with the reference at 630 nm. Standard curves were constructed using ChroMate® 4300 Manager software (Awareness Technology Inc.). Each experiment was performed \geq 3 times.

Nitrate (NO₃⁻) reductase assay. In the cell, NO undergoes a series of reactions with numerous molecules present in biological fluids and is eventually metabolized to nitrite (NO₂⁻) and NO₃⁻ (18). At 4, 8, 12 and 24 h post-infection, cells of all groups were centrifuged at 800 x g for 20 min at 4°C to collect the supernatant (1 ml) and were stored at -80°C. After 30 min, the supernatant samples were assayed for NO levels using a Microplate Assay from Active Motif (Carlsbad, CA, USA) and a NO Quantitation kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Each experiment was performed in triplicate.

Western blot assay. Cells in each treatment group were centrifuged at 300 x g for 5 min at 4°C, followed by incubation with radioimmunoprecipitation assay lysis buffer (Beyotime

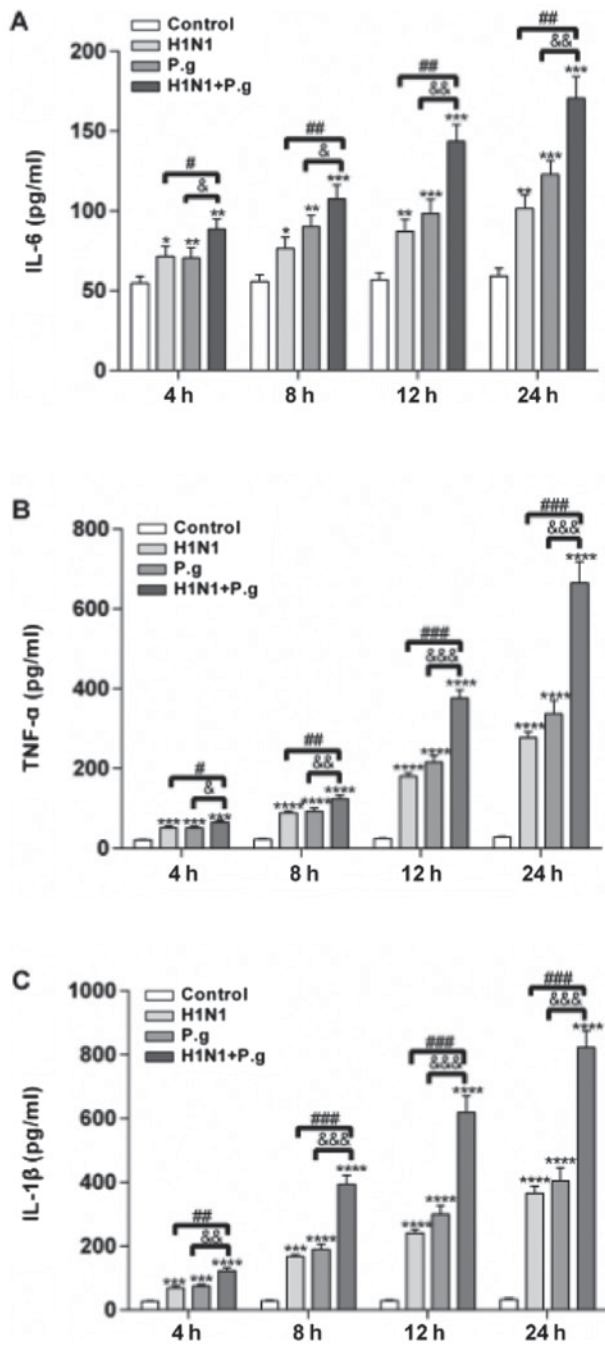


Figure 1. Effects of P.g. on the expression levels of TNF- α , IL-1 β and IL-6 in BEAS-2B cells infected with influenza virus. The concentrations of (A) IL-6, (B) TNF- α and (C) IL-1 β in the control, H1N1, P.g. and H1N1+P.g. groups following infection of BEAS-2B for 4, 8, 12 and 24 h, were detected by ELISA. The values are presented as the mean \pm standard deviation. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. control group at the same time point; #P<0.05, ##P<0.01 and ###P<0.001 vs. H1N1 group at the same time point; &P<0.05, &#P<0.01 and &&#P<0.001 vs. P.g. group at the same time point. IL, interleukin; H1N1, influenza 1 virus H1N1; P.g., *Porphyromonas gingivalis*; TNF- α , tumor necrosis factor- α .

Institute of Biotechnology) on ice. Protein concentration was quantified with a Bradford assay. Then, 50 mg total protein extracts were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA), followed by blocking for 1 h at room temperature in blocking buffer (cat. no. P0023B; Beyotime Institute of Biotechnology). The membranes were incubated

with the following primary antibodies overnight at 4°C: Rabbit anti-inducible NO synthase (iNOS; cat. no. ab3523; 1:1,000; Abcam, Cambridge, MA, USA), rabbit anti-B-cell lymphoma-2 (Bcl-2; cat. no. ab196495; 1:1,000), rabbit anti-Bcl-2-associated X protein (Bax; cat. no. ab53154; 1:1,000), rabbit anti-caspase-3 (cat. no. ab4051; 1:1,000) and mouse anti-GAPDH (cat. no. ab8245; 1:1,000) or mouse anti- β -actin (cat. no. ab6276; 1:1,000; all Abcam). Following subsequent incubation with goat anti-rabbit (cat. no. ab205718) or goat anti-mouse (cat. no. ab205719) horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. bands were detected using an Enhanced Chemiluminescence kit (Beyotime Institute of Biotechnology). Image J software version 1.48 (National Institutes of Health, Bethesda, MD, USA) was used to analyze relative protein band density. iNOS was measured at 4, 8, 12 and 24 h post-infection; Bax, Bcl-2 and caspase-3 were measured at 24 h post-infection only. Each sample was analyzed in triplicate.

Apoptosis analysis. Treated cells were collected by low-speed centrifugation (300 x g, for 5 min at 4°C), and cell pellets were re-suspended in 1 ml PBS solution. Apoptosis was analyzed using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. In brief, cells were washed, centrifuged (300 x g, 4°C, 5 min) and re-suspended in PI staining buffer containing 50 μ l/ml PI. The cell mixture was subsequently incubated at 4°C for 30 min in a dark environment and stained with 5 μ l Annexin V-FITC to detect apoptosis with a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). FlowJo software 7.6.5 (Tree Star, Inc., Ashland, OR, USA) was used to analyze the results of the flow cytometry. All experiments were performed in triplicate.

Statistical analysis. All of the results were expressed as the mean \pm standard deviation and all statistical analyses were performed using the SPSS 18.0 version (SPSS, Inc., Chicago, IL, USA). Statistical comparisons were conducted using one-way analysis of variance followed by Dunnett's post-hoc test for multiple comparisons. Non-linear regression models were employed to calculate the concentration of each cytokine in the culture supernatants. P<0.05 was considered to indicate a statistically significant difference.

Results

P. gingivalis promotes the secretion of TNF- α , IL-1 β and IL-6 from BEAS-2B cells infected with H1N1. To study the effects of *P. gingivalis* on the secretion of TNF- α , IL-1 β and IL-6 from BEAS-2B cells infected with H1N1, ELISAs were performed to detect their concentrations at various time points (4, 8, 12 and 24 h) in all groups. The results of the present study revealed that following infection of BEAS-2B cells with *P. gingivalis* and H1N1, H1N1 only or *P. gingivalis* alone, the concentrations of TNF- α , IL-1 β and IL-6 in the supernatant were significantly increased at each time point, when compared with the control group (at 4, 8, 12 and 24 h; Fig. 1). Following BEAS-2B cell infection with *P. gingivalis* and H1N1, the concentrations of TNF- α , IL-1 β and IL-6 in the supernatant

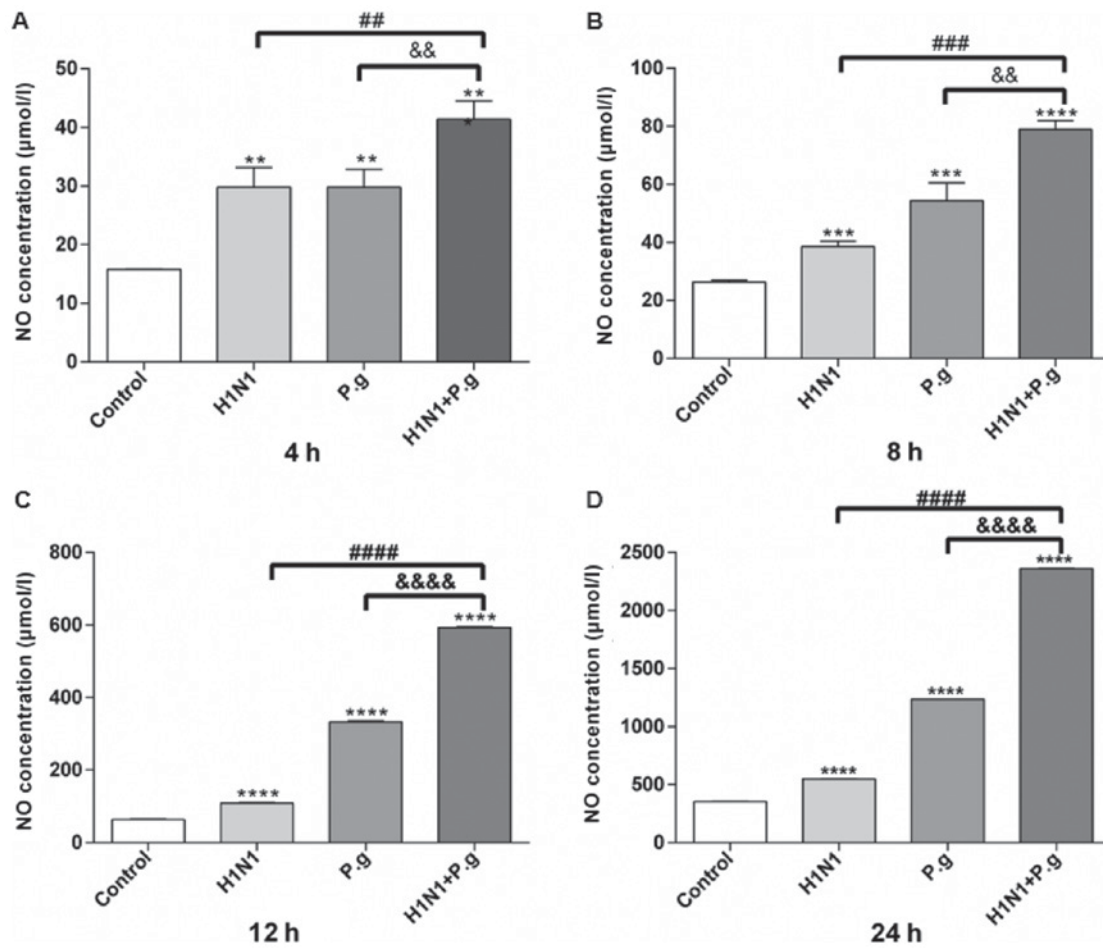


Figure 2. Effects of *P.g.* on the NO expression levels in BEAS-2B cells infected with influenza virus. The expression levels of NO in the control, H1N1, *P.g.* and H1N1+ *P.g.* groups following BEAS-2B cells infected for (A) 4, (B) 8, (C) 12 and (D) 24 h were detected by a nitrate reductase assay. The values represent the mean \pm standard deviation. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ vs. control group; ## $P < 0.01$, ### $P < 0.001$ and #### $P < 0.0001$ vs. H1N1 group; && $P < 0.01$ and &&&& $P < 0.0001$ vs. *P.g.* group. H1N1, influenza 1 virus H1N1; *P.g.*, *Porphyromonas gingivalis*; NO, nitric oxide.

were significantly increased at each time point, compared with the H1N1 and *P. gingivalis* alone groups (at 4, 8, 12 and 24 h; Fig. 1). These results demonstrated that lung epithelial cells infected with H1N1 and *P. gingivalis* promoted the production of inflammatory cytokines.

P. gingivalis increases the NO expression levels in BEAS-2B cells infected with H1N1. In order to observe the effects of *P. gingivalis* on the NO expression levels in BEAS-2B cells infected with H1N1, a NO_3^- reductase assay was performed to determine the NO expression levels at various time points in all groups. The results of the present study demonstrated that following the infection of BEAS-2B cells with *P. gingivalis* and H1N1, H1N1 only or *P. gingivalis* alone, the expression levels of NO in the supernatant significantly increased at each time point in all groups, when compared with the control group (Fig. 2). In addition, BEAS-2B cells infected with *P. gingivalis* and H1N1 exhibited significantly increased expression levels of NO in the supernatant at each time point when compared with the H1N1 only and *P. gingivalis* alone groups (at 4, 8, 12 and 24 h; Fig. 2A-D). These results indicated that *P. gingivalis* may have promoted the production of NO in lung epithelial cells infected with influenza virus H1N1.

P. gingivalis increases the protein expression levels of iNOS in BEAS-2B cells infected with H1N1. To further confirm the effects of *P. gingivalis* on the protein expression of iNOS in BEAS-2B cells infected with H1N1, western blot analysis was conducted. The present study reported that the BEAS-2B cells infected with *P. gingivalis* and H1N1, H1N1 only or *P. gingivalis* alone exhibited significantly increased protein expression levels of iNOS at each time point when compared with the control group (at 4, 8, 12 and 24 h; Fig. 3A and B). In addition, BEAS-2B cells infected with *P. gingivalis* and H1N1 exhibited significantly increased protein expression levels of iNOS at each time point when compared with the H1N1 and *P. gingivalis* only groups (at 4, 8, 12 and 24 h; Fig. 3A and B), indicating that lung epithelial cells co-infected with H1N1 and *P. gingivalis* may have further induced the expression of iNOS.

P. gingivalis increases the BEAS-2B cellular apoptosis rate following infection with H1N1. To further study the effects of *P. gingivalis* on the apoptosis rate of BEAS-2B cells infected with H1N1, flow cytometric analysis was conducted. The results of the present study demonstrated that BEAS-2B cells infected with *P. gingivalis* and H1N1, H1N1 only or *P. gingivalis* alone, exhibited significantly increased rates of apoptosis at each time point compared with the Control group

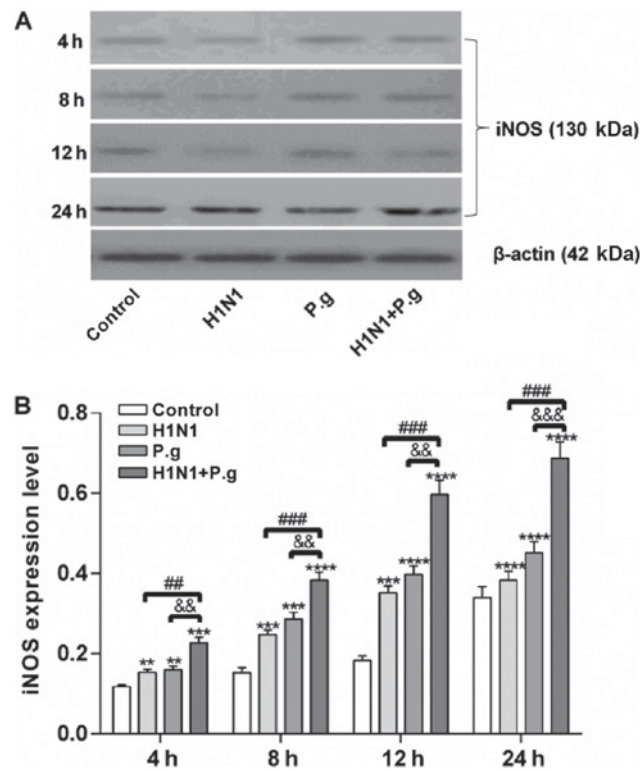


Figure 3. Effects of P.g. on the protein expression levels of iNOS in BEAS-2B cells infected with influenza virus. (A) Protein expression levels of iNOS in the control, H1N1, P.g. and H1N1+ P.g. groups following infection of BEAS-2B cells for 4, 8, 12 and 24 h, detected by western blot analysis. (B) Relative quantification of western blot analysis depicted in the bar graphs. The values are presented as the mean \pm standard deviation. **P<0.01, ***P<0.001 and ****P<0.0001 vs. control group at the same time point; ##P<0.01 and ###P<0.001 vs. H1N1 group at the same time point; &&P<0.01 and &&&P<0.001 vs. P.g. group at the same time point. H1N1, influenza 1 virus H1N1; iNOS, inducible nitric oxide synthase; P.g., *Porphyromonas gingivalis*.

(at 4, 8, 12 and 24 h; Fig. 4A-E). Additionally, BEAS-2B cells infected with *P. gingivalis* and H1N1 exhibited a significantly increased apoptotic rate at each time point when compared with the H1N1 and *P. gingivalis* only groups (at 4, 8, 12 and 24 h; Fig. 4A-E). These results revealed that *P. gingivalis* may have induced an inflammatory response and subsequently promoted the induction of apoptosis.

Effects of P. gingivalis on the Bcl-2/Bax/caspase-3 signaling pathway in BEAS-2B cells infected with influenza virus. The Bcl-2 and caspase families serve an important role in the regulation of cellular apoptosis (19). In order to characterize the mechanism by which the co-infection with *P. gingivalis* and H1N1 induces BEAS-2B cellular apoptosis, the protein expression levels of Bcl-2, Bax and caspase-3 were detected by western blotting. Compared with the control, the relative protein expression levels of caspase-3 and Bax were significantly increased, and the relative expression of Bcl-2 protein decreased significantly in lung epithelial cells following co-infection with *P. gingivalis* and H1N1 for 24 h (Fig. 5A and B). These results revealed that co-infection with *P. gingivalis* and H1N1 may have promoted cellular apoptosis in a manner associated with the regulation of the Bcl-2/Bax/caspase-3 signaling pathway.

Discussion

Periodontal disease is a chronic inflammatory process associated with infection with *P. gingivalis*, causing a local and

systemic inflammatory response (20). Numerous studies have reported that periodontal disease may be mediated by *P. gingivalis* and systemic diseases, including cardiovascular and cerebrovascular diseases, diabetes and respiratory infections (21-23). Oropharyngeal secretions enter the human respiratory system via breathing and swallowing mechanisms, and may therefore lead to pulmonary disease associated with periodontal pathogens such as *P. gingivalis* (24). The influenza virus, such as H1N1, is a common pathogen causing lung infection in humans (25). Clinical research suggests that infection of lung epithelial cells by *P. gingivalis* or influenza viruses does not induce apoptosis and necrosis of respiratory epithelial cells in a short period of time (26,27). However, upon co-infection with *P. gingivalis* and influenza viruses, a large proportion of apoptotic and necrotic respiratory epithelial cells were observed within a short period (28). In the present study, using lung epithelial cells co-infected with *P. gingivalis* and the influenza virus H1N1, the expression levels of inflammatory cytokines and NO were detected to investigate the mechanism underlying the association between periodontal disease and respiratory infection.

NOS catalyzes the synthesis of NO *in vivo*; under normal physiological states, NO synthesis mediated by endothelial NOS serves a major role in the regulation of vascular endothelial cells (29). Low or absent expression of iNOS in endothelial cells leads to the secretion of large amounts of NO, and the expression of iNOS increases correspondingly following severe infection (30,31). Cytotoxic products, such as lipopolysaccharide, of *P. gingivalis* induce host cells to

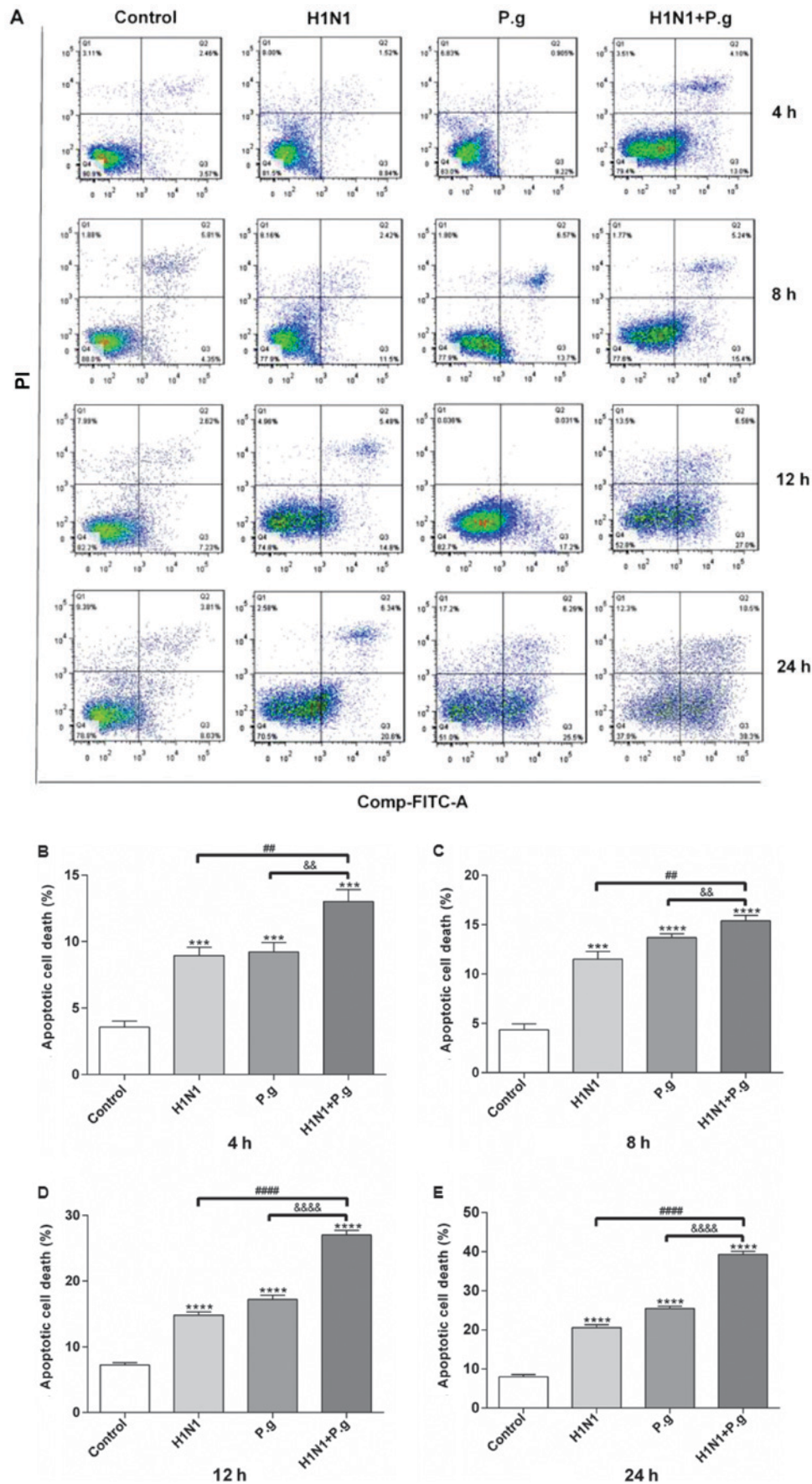


Figure 4. Effects of *P.g.* on the cell apoptosis rate in BEAS-2B cells infected with influenza virus. (A) BEAS-2B cell apoptosis in the control, H1N1, *P.g.* and H1N1+ *P.g.* groups following infection of BEAS-2B cells for 4, 8, 12 and 24 h, detected by flow cytometry analysis. The apoptotic rates of BEAS-2B cells in the control, H1N1, *P.g.* and H1N1+ *P.g.* groups following infection of BEAS-2B for (B) 4, (C) 8, (D) 12 and (E) 24 h, as detected by flow cytometry analysis. The values are presented as the mean \pm standard deviation. *** P <0.001 and **** P <0.0001 vs. control group; ** P <0.01, and ### P <0.0001 vs. H1N1 group; && P <0.01 and &&& P <0.0001 vs. *P.g.* group. H1N1, influenza 1 virus H1N1; FITC, fluorescein isothiocyanate; PI, propidium iodide; *P.g.*, *Porphyromonas gingivalis*.

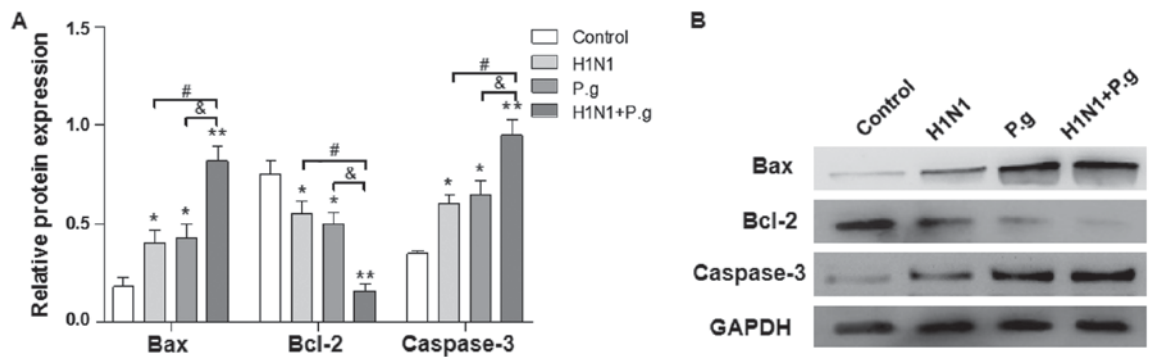


Figure 5. Effects of P.g. on the Bcl-2/Bax/caspase-3 signaling pathway in BEAS-2B cells infected with influenza virus. (A) Relative protein expression of Bcl-2, Bax and caspase-3 in the control, H1N1, P.g. and H1N1+ P.g. groups following the infection of BEAS-2B cells for 24 h, was (B) detected by western blot analysis. The values are presented as the mean \pm standard deviation. *P<0.05 and **P<0.01 vs. control group. #P<0.05 vs. H1N1 group; &P<0.05 vs. P.g. groups. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; H1N1, influenza 1 virus H1N1; P.g., *Porphyromonas gingivalis*.

release inflammatory cytokines, of which TNF- α , IL-1 β and IL-6 are of the most importance (32,33). *P. gingivalis*-infected cells expressed TNF- α , IL-1 β and IL-6, along with other inflammatory cytokines; therefore, the synthesis of NOS is induced to increase the production of NO, which leads to endothelial cell dysfunction (34). Research suggests that excess NO production may be closely associated with the invasion of vascular endothelial cells by *P. gingivalis* (18). NO production may cause cytotoxicity and mediate pulmonary epithelial cell damage (19,35,36). The results of the present study revealed that *P. gingivalis* may induce the production of a large number of inflammatory cytokines in lung epithelial cells. It was hypothesized that in the present study, *P. gingivalis* may have induced an inflammatory reaction in lung epithelial cells, promoting the accumulation of NO, which may have resulted in damage to lung epithelial cells and the induction of apoptosis. When compared with the control group, and the viral and bacterial infection groups, apoptotic cell numbers increased by the 4 h time point, and the highest levels were observed at 24 h in the group co-infected with *P. gingivalis* and H1N1; the percentage of apoptotic lung epithelial cells corresponded to the increase in NO production. In the present study, *P. gingivalis* may have promoted the production of NO in lung epithelial cells infected with influenza virus H1N1, which may have led to an increase in the inflammatory reaction and lung epithelial cell injury.

Regarding the characterization of the mechanism by which the co-infection of *P. gingivalis* and H1N1 induces BEAS-2B cellular apoptosis, the present study reported that compared with the control, the relative protein expression levels of caspase-3 and Bax increased significantly, and that the relative expression levels of Bcl-2 protein decreased significantly, within 24 h in BEAS-2B cells following co-infection with *P. gingivalis* and H1N1. The results of the present study revealed that co-infection with *P. gingivalis* and H1N1 may have promoted cellular apoptosis by modulating the Bcl-2/Bax/caspase-3 signaling pathway.

In conclusion, the present study demonstrated that lung epithelial cells infected with H1N1 and *P. gingivalis* led to the promoted production of inflammatory cytokines and the expression of iNOS, which may have also increased the accumulation of NO, resulting in an increased

proportion of lung epithelial cells undergoing apoptosis via the Bcl-2/Bax/caspase-3 signaling pathway. The present study provided the experimental foundation for research into periodontal pathogens in the injury and apoptosis of lung epithelial cells, in order to confirm whether there is an association between periodontal disease and respiratory infection.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YP and XL designed the study. YC, RZ, ZY, YL, YF, YZ and PL performed the experiments. YC analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Jin LJ, Armitage GC, Klinge B, Lang NP, Tonetti M and Williams RC: Global oral health inequalities: Task group-periodontal disease. *Adv Dent Res* 23: 221-226, 2011.
- Noguchi S, Toyokawa S, Miyoshi Y, Suyama Y, Inoue K and Kobayashi Y: Five-year follow-up study of the association between periodontal disease and myocardial infarction among Japanese male workers: MY Health Up study. *J Public Health (Oxf)* 37: 605-611, 2015.
- Chapple IL and Genco R; Working group 2 of the joint EFP/AAP workshop: Diabetes and periodontal diseases: Consensus report of the Joint EFP/AAP workshop on periodontitis and systemic diseases. *J Periodontol* 84 (4 Suppl): S106-S112, 2013.
- Mikuls TR, Payne JB, Yu F, Thiele GM, Reynolds RJ, Cannon GW, Markt J, McGowan D, Kerr GS, Redman RS, *et al.*: Periodontitis and *porphyromonas gingivalis* in patients with rheumatoid arthritis. *Arthritis Rheumatol* 66: 1090-1100, 2014.
- Fernández-Plata R, Olmedo-Torres D, Martínez-Briseño D, García-Sancho C, Franco-Marina F and González-Cruz H: Prevalence of severe periodontal disease and its association with respiratory disease in hospitalized adult patients in a tertiary care center. *Gac Med Mex* 151: 608-613, 2015 (In Spanish).
- Cho BH, Jung YH, Kim DJ, Woo BH, Jung JE, Lee JH, Choi YW and Park HR: Acetylshikonin suppresses invasion of *Porphyromonas gingivalis*-infected YD10B oral cancer cells by modulating the interleukin-8/matrix metalloproteinase axis. *Mol Med Rep* 17: 2327-2334, 2018.
- Anand PS, Kamath KP and Anil S: Role of dental plaque, saliva and periodontal disease in *Helicobacter pylori* infection. *World J Gastroentero* 20: 5639-5653, 2014.
- Sharma N and Shamsuddin H: Association between respiratory disease in hospitalized patients and periodontal disease: A cross-sectional study. *J Periodontol* 82: 1155-1160, 2011.
- Bousbia S, Papazian L, La Scola B and Raoult D: Detection of plant DNA in the bronchoalveolar lavage of patients with ventilator-associated pneumonia. *PLoS One* 5: e11298, 2010.
- El-Rabbany M, Zaghlol N, Bhandari M and Azarpazhooh A: Prophylactic oral health procedures to prevent hospital-acquired and ventilator-associated pneumonia: A systematic review. *Int J Nurs Stud* 52: 452-464, 2015.
- Bansal M, Khatri M and Taneja V: Potential role of periodontal infection in respiratory diseases-a review. *J Med Life* 6: 244-248, 2013.
- Lee SY, Bae CS, Seo JH, Cho SS, Bae MS, Oh DS and Park DH: Mycoleptodonoides aitchisonii suppresses asthma via Th2 and Th1 cell regulation in an ovalbumin-induced asthma mouse model. *Mol Med Rep* 17: 11-20, 2018.
- Liu Y, Xie L, Yang M, Tan X, Zeng Y, Zheng G, Chen Y and Chen P: PPAR- α improves the recovery of lung function following acute respiratory distress syndrome by suppressing the level of TGF- β 1. *Mol Med Rep* 16: 49-56, 2017.
- Liu B, Wang J, Cheng L and Liang J: Role of JNK and NF- κ B pathways in *Porphyromonas gingivalis* LPS-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells. *Mol Med Rep* 8: 1594-1600, 2013.
- Chambers C, Skowronski DM, Sabaiduc S, Winter AL, Dickinson JA, De Serres G, Gubbay JB, Drews SJ, Martineau C, Eshaghi A, *et al.*: Interim estimates of 2015/16 vaccine effectiveness against influenza A(H1N1)pdm09, Canada, February 2016. *Euro Surveill* 21: 30168, 2016.
- Wang J and Kubers P: A reservoir of mature cavity macrophages that can rapidly invade visceral organs to affect tissue repair. *Cell* 165: 668-678, 2016.
- Shah NS, Greenberg JA, McNulty MC, Gregg KS, Riddell J IV, Mangino JE, Weber DM, Hebert CL, Marzec NS, Barron MA, *et al.*: Bacterial and viral co-infections complicating severe influenza: Incidence and impact among 507 U.S. patients, 2013-14. *J Clin Virol* 80: 12-19, 2016.
- Alayan J, Ivanovski S, Gemmell E, Ford P, Hamlet S and Farah CS: Deficiency of iNOS contributes to *Porphyromonas gingivalis*-induced tissue damage. *Oral Microbiol Immunol* 21: 360-365, 2006.
- Ahamed M, Akhtar MJ, Khan MAM, Alhadlaq HA and Aldalbahi A: Nanocubes of indium oxide induce cytotoxicity and apoptosis through oxidative stress in human lung epithelial cells. *Colloids Surf B Biointerfaces* 156: 157-164, 2017.
- Gawron K, Montgomery A, Łazarz-Bartyzel K, Bereta G, Chomyszyn-Gajewska M, Venables P and Potempa J: *Porphyromonas gingivalis* peptidyl arginine deiminase: A Unique bacterial PAD with implications for periodontal disease and rheumatoid Arthritis. *Protein Deimination Human Health Dis*: 99-135, 2017.
- Olsen I and Yilmaz Ö: Modulation of inflammasome activity by *Porphyromonas gingivalis* in periodontitis and associated systemic diseases. *J Oral Microbiol* 8: 30385, 2016.
- Igari K, Inoue Y and Iwai T: An experimental model of peripheral vascular disease involving the intravenous injection of oral bacteria. *Ann Vasc Dis* 9: 267-271, 2016.
- Card JW, Carey MA, Voltz JW, Bradbury JA, Ferguson CD, Cohen EA, Schwartz S, Flake GP, Morgan DL, Arbes SJ Jr, *et al.*: Modulation of allergic airway inflammation by the oral pathogen *Porphyromonas gingivalis*. *Infect Immun* 78: 2488-2496, 2010.
- Okuda K, Kimizuka R, Abe S, Kato T and Ishihara K: Involvement of periodontopathic anaerobes in aspiration pneumonia. *J Periodontol* 76 (11 Suppl): S2154-S2160, 2005.
- Guo L, Feng K, Wang YC, Mei JJ, Ning RT, Zheng HW, Wang JJ, Worthen GS, Wang X, Song J, *et al.*: Critical role of CXCL4 in the lung pathogenesis of influenza (H1N1) respiratory infection. *Mucosal Immunol* 10: 1529-1541, 2017.
- Porto AN, Cortelli SC, Borges AH, Matos FZ, Aquino DR, Miranda TB, Oliveira Costa F, Aranha AF and Cortelli JR: Oral and endotracheal tubes colonization by periodontal bacteria: A case-control ICU study. *Eur J Clin Microbiol Infect Dis* 35: 343-351, 2016.
- Li Q, Pan C, Teng D, Lin L, Kou Y, Haase EM, Scannapieco FA and Pan Y: *Porphyromonas gingivalis* modulates *Pseudomonas aeruginosa*-induced apoptosis of respiratory epithelial cells through the STAT3 signaling pathway. *Microbes Infect* 16: 17-27, 2014.
- Connolly E, Millhouse E, Doyle R, Culshaw S, Ramage G and Moran GP: The *Porphyromonas gingivalis* haemagglutinins HagB and HagC are major mediators of adhesion and biofilm formation. *Mol Oral Microbiol* 32: 35-47, 2017.
- Chuaiphichai S, Crabtree MJ, McNeill E, Hale AB, Trelfa L, Channon KM and Douglas G: A key role for tetrahydrobiopterin-dependent endothelial NOS regulation in resistance arteries: Studies in endothelial cell tetrahydrobiopterin-deficient mice. *Brit J Pharmacol* 174: 657-671, 2017.
- Assis MC, Freitas C, Saliba AM, D'A Carvalho AP, Simao TA, Albano RM and Plotkowski MC: Up-regulation of Fas expression by *Pseudomonas aeruginosa*-infected endothelial cells depends on modulation of iNOS and enhanced production of NO induced by bacterial type III secreted proteins. *Int J Mol Med* 18: 355-363, 2006.
- Yan M, Hou M, Liu J, Zhang S, Liu B, Wu X and Liu G: Regulation of iNOS-derived ROS generation by HSP90 and Cav-1 in porcine reproductive and respiratory syndrome virus-infected swine lung injury. *Inflammation* 40: 1236-1244, 2017.
- Ke X, Lei L, Li H, Li H and Yan F: Manipulation of necroptosis by *Porphyromonas gingivalis* in periodontitis development. *Mol Immunol* 77: 8-13, 2016.
- Yiemwattana I and Kaomongkolgit R: Alpha-mangostin suppresses IL-6 and IL-8 expression in *P. gingivalis* LPS-stimulated human gingival fibroblasts. *Odontology* 103: 348-355, 2015.
- Wu C, Guo S, Niu Y, Yang L, Liu B, Jiang N, Su M and Wang L: Heat-shock protein 60 of *Porphyromonas gingivalis* may induce dysfunction of human umbilical endothelial cells via regulation of endothelial-nitric oxide synthase and vascular endothelial-cadherin. *Biomed Rep* 5: 243-247, 2016.
- Issaad N, Aitlounis A and Larabadjebari F: Cytotoxicity and actin cytoskeleton damage induced in human alveolar epithelial cells by *Androctonus australis* hector venom. *Toxin Rev* 37: 67-74, 2017.
- Kaviyarasu K, Geetha N, Kanimozhi K, Maria Magdalane C, Sivaranjani S, Ayeshamariam A, Kennedy J and Maaza M: In vitro cytotoxicity effect and antibacterial performance of human lung epithelial cells A549 activity of Zinc oxide doped TiO₂ nanocrystals: Investigation of bio-medical application by chemical method. *Mater Sci Eng C Mater Biol Appl* 74: 325-333, 2017.

