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Bacterial-killing Effect of Atmospheric Pressure Non-equilibrium Plasma Jet and Oral Mucosa Response*

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Summary: Recently, plasma sterilization has attracted increasing attention in dental community for the atmospheric pressure non-equilibrium plasma jet (APNPs), which is driven by a kilohertz pulsed DC power, may be applied to the dental and oral diseases. However, it is still in doubt whether APNPs can effectively kill pathogenic bacteria in the oral cavity and produce no harmful effects on normal oral tissues, especially on normal mucosa. The aim of this study was to evaluate the bacterial-killing effect of APNPs in the biofilms containing a single breed of bacteria (*Porphyromonas gingivalis*, *P.g.*), and the pathological changes of the oral mucosa after treatment by APNPs. *P.g.* was incubated to form the biofilms *in vitro*, and the samples were divided into three groups randomly: group A (blank control); group B in which the biofilms were treated by APNPs (the setting of the equipment: 10 kHz, 1600 ns and 8 kV); group C in which the biofilms were exposed only to a gas jet without ignition of the plasma. Each group had three samples and each sample was processed for up to 5 min. The biofilms were then fluorescently stained, observed and photographed under a laser scanning confocal microscope. In the animal experiment, six male Japanese white rabbits were divided into two groups randomly (n=3 in each group) in terms of the different post-treatment time (1-day group and 5-day group). The buccal mucosa of the left side and the mucosa of the ventral surface of the tongue were treated by APNPs for 10 min in the same way as the bacterial biofilm experiment in each rabbit, and the corresponding mucosa of the other sides served as normal control. The clinical manifestations of the oral mucosa were observed and recorded every day. The rabbits were sacrificed one or five day(s) after APNPs treatment. The oral mucosa were harvested and prepared to haematoxylin and eosin-stained sections. Clinical observation and histopathological scores were used to assess mucosal changes. The results showed the obvious *P.g.* biofilms were formed at 10 days, and most of the bacteria in groups A and C were alive under a laser scanning confocal microscope, but the bacteria in the group B were almost all dead. In animal experiment, no ulcers, anabrosis and oral mucositis were found in both the 1-day and 5-day groups. The average mucous membrane irritation index was –0.83 and –0.67 in the 1-day and 5-day groups, respectively, suggesting that no intense mucosal membrane irritation responses occurred. It was concluded that APNPs could effectively kill *P.g.* in the biofilms and did not cause any pathological changes in the normal mucosa, suggesting that the plasma jet (APNPs) may be applied to oral diseases as a novel sterilization device in the future.

Key words: plasma jet; sterilization; *porphyromonas gingivalis*; biofilm; oral mucosa

Most dental disorders are related to pathogenic bacteria. Periodontal disease, one of the most common oral diseases, is found to be correlated with special bacteria under the gingival plaque. *Porphyromonas gingivalis* (*P.g.*) is a gram-negative, nonmotile, pleomorphic rod and an obligate anaerobe, which is believed to be the most important infectious agent for periodontal disease[1, 2]. This bacteria is not only seen in subgingival plaque of healthy individuals of all age groups as part of the indigenous supra- and sub-gingival microflora[3, 4], but also found in large quantity in chronic periodontitis patients, especially at sites where bleeding occurs[5].

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The current treatments for periodontal disease consist of mechanical method, drug therapy, laser therapy, etc., but they all have their own limitations. For example, the mechanical method is prone to damage the surface of the teeth[6]. As an adjuvant therapy, drug therapy tends to induce drug resistance. Laser therapy is a new treatment approach, but it has many side effects and the cost of this treatment is relatively high[7]. Furthermore, after root canal therapy, patients will possibly develop apical periodontitis due to existent bacteria that can't be killed completely by medications in practice, which often results in the failure of root canal therapy[8, 9]. Taken together, an effective alternative for local sterilization has been clinically desired.

Numerous studies reported that plasmas, an innovative sterilization device, can easily access into narrow and confined spaces and has a capability of eliminating various bacteria in the oral cavity[10–16], and the atmospheric pressure, non-equilibrium plasma jet (APNPs) holds promise for the treatment of dental disorders. It has such advantages as no risk of puncturing the oral cavity, ability to manually operate at room temperature, good safety, low gas temperature. However, it is still uncertain of the effect of APNPs on periodontal pathogens and the biosecurity of this device. In this study, the effects of APNPs on *P.g.* in the biofilms, and the normal oral mucosa were examined with an attempt to evaluate the killing effect of APNPs on oral bacteria and the safety of the device.

1 MATERIALS AND METHODS

1.1 APNPs

The APNPs device was described in detail in some literatures previously[17–20]. Briefly, helium and oxygen mixture (He/O_2) was injected into a hollow barrel and the HV pulsed DC voltage (voltage up to 10 kV, frequency to 10 kHz, and pulse width varying from 200 ns to DC) was applied to HV electrodes. In this study the pulse frequency of 10 kHz, pulse width t_{pw} of 1600 ns, and applied voltage V of 8 kV were used. Diameters of the syringe and syringe nozzle were 6 mm and 0.7 mm, respectively. Inner diameter of the needle was 200 μm, and the length was 3 cm, while the distance between the jet nozzle and the top surface of treated object was kept at 10 mm.

1.2 Bacterial Strains and Culture Conditions

P.g. (ATCC 33277) were obtained from the American Type Culture Collection (ATCC, USA) and stored at frozen temperature of about –80°C prior to use. The bacterial strains were grown on anaerobic blood agar plates or broth (Department of Clinical Laboratory, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China) in an anaerobic bag (GENbag, France) at 37°C. The bacterial strains were maintained on the anaerobic blood agar plates in the anaerobic bag at 4°C prior to use.

1.3 Animals and Animal Maintenance

Animal maintenance was carried out in accordance with the Law of People's Republic of China on the Protection of Animals and the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

Six male 5-month-old Japanese white rabbits (weighing 1500–2000 g, provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, China) were quarantined for 7 days to determine whether their oral mucosa and tongues were normal or not. Animals with normal mucosa were distributed randomly into two groups. In order to facilitate identification, each rabbit's tail was marked, and housed individually (6 cages in total). Food and tap water were available *ad libitum*.

1.4 Development of Mono-species Biofilms

P.g. was grown on the anaerobic blood agar plates for 5 days at 37°C, then a colony was picked up to prepare bacterial suspension with normal saline and the absorbance (*A* value) was adjusted to 0.1±0.05 at a wavelength of 600 nm, which corresponded to a bacterial cell count of 10^8 CFU/mL. The bacterial suspension was inoculated on sterile cover slips placed in a 24-well plate. A total of 1 mL of bacterial suspension and 4 mL of broth were transferred into each well, and incubated at 37°C in the anaerobic bag. The bacteria were incubated alone to establish mono-species biofilms.

After incubation, the liquid inside each well was removed and 5 mL of fresh culture medium was added every 5 days. On the 3rd, 5th, 7th, 10th, 12th and 15th day, the medium of the three wells was removed respectively. Subsequently, the wells were washed gently with normal saline three times, and then stained and detected by using the BacLight bacterial viability assay kit (Invitrogen, USA) according to the manufacturer's instructions. The cover slip carrying biofilm in each cell well was taken out, and covered by another cover slip. The cover slips were observed under a laser scanning confocal microscope (Olympus Fv 500, Japan) and 3D images of the mono-species biofilms were obtained by using software.

1.5 Study Design

1.5.1 *P.g.* Biofilms Experiment After *P.g.* formed apparent mono-species biofilms, the biofilms were divided into three groups at random with 3 samples in each group: group A (blank control), group B in which the biofilms were treated by APNPs (fig. 1), and group C in which the samples were exposed only to a gas jet without ignition of the plasma. The culture medium of each cell well was removed and washed gently with normal saline three times. Each sample was processed for up to 5 min, stained and observed under a laser scanning confocal microscope.

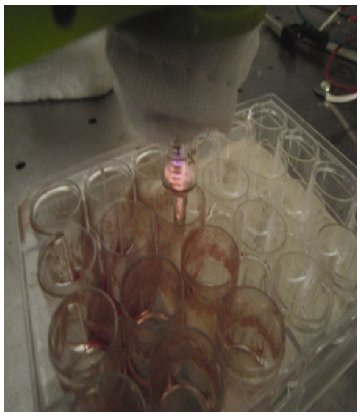

Fig. 1 A photograph of APNPs treating the biofilms on the cover slips placed in a 24-well plate

1.5.2 Animal Experiment The glossodesmus and the left maxillary lip cleft of each rabbit were well fixed, ensuring the operation on the buccal mucosa of the left side and the mucosa of the ventral surface of the tongue (approximately 0.5 cm× 0.5 cm of each). The corresponding mucosa of the other side served as control. The aforementioned mucosa of rabbits was treated by APNPs for 10 min under general anesthesia (fig. 2). The mucosa of the rabbits was observed once daily. Three rabbits in 1-day or 5-day groups were sacrificed by air embolism one or five day(s) after APNPs treatment. The treated and control mucosa tissues were removed. After fixation in 10% neutral buffered formalin for 24 h at 4°C, all tissues were embedded in paraffin and sectioned 4–6 μm in thickness. The sections were stained with hematoxylin and eosin (H&E) for microscopic examination and evaluation.

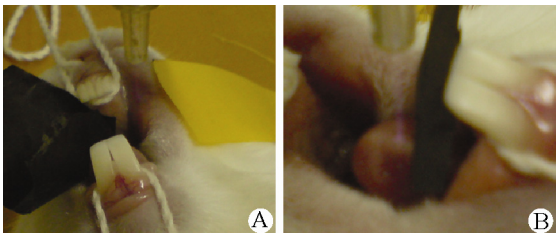

Fig. 2 Fixation of a rabbit on the surgery table under general anesthesia, and treatment by APNPs (10 kHz, 1600 ns and 8 kV)
A: The buccal mucosa of the left side; B: The mucosa of the ventral surface of the tongue

1.6 Evaluation

1.6.1 Biofilms Experiment The viability of *P.g.* in the biofilms was analyzed after nucleic acid staining with SYTO 9 and propidium iodide (PI) by the laser scanning confocal microscopy. SYTO9 (green fluorescence) is able to enter all cells and is used for total cell counting, while PI (red fluorescence) enters only cells with damaged cytoplasmic membranes. SYTO 9 fluorescence was detected by excitement at 488 nm, and emission at bandwidth of 500–550 nm. PI fluorescence was detected by excitement at 488 nm, and emission at 560 nm. All experiments were repeated three times.

1.6.2 Animal Experiment Clinical manifestations such as hyperemia, swelling, ulcer or anabrosis were evaluated. Histopathological changes of normal mucosa were assessed by three pathologists separately according to the standards established on the vaginal mucous irritation test[21]. All of the specimens were examined in a double-blind manner.

2 RESULTS

2.1 Killing Effect of APNPs on *P.g.*

Laser scanning confocal microscopy revealed apparent 3D images of mono-species biofilms after *P.g.* was incubated for 10 days (fig. 3). In the control groups (groups A and C), bacterial cells were tightly arranged, and all cells exhibited green fluorescence except few doddery bacteria shown yellow or red. After treatment by APNPs (group B), most bacterial cells presented yellow or red fluorescence (fig. 4).

2.2 Effect of APNPs on Normal Mucosa

After treatment by APNPs, the buccal mucosa of the left side and the mucosa of the ventral surface of the tongue did not show any hyperemia, swelling, ulcer or anabrosis. Histopathological results (fig. 5) showed that the average mucous membrane irritation index of the 1-day group and the 5-day group was –0.83 and –0.67 respectively, suggesting that both groups had no apparent mucous membrane irritation responses, and APNPs could not induce mucous membrane irritation.

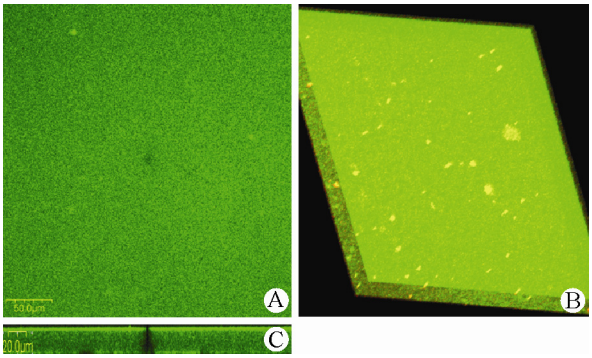

Fig. 3 Images of mono-species biofilms formed by *P.g.* under a laser scanning confocal microscope (×40)
A: 2D image; B: 3D image; C: Deep image

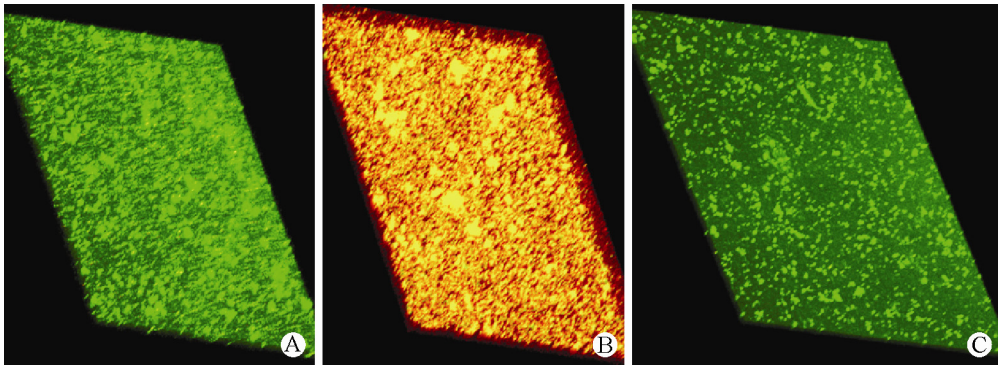

Fig. 4 Laser scanning confocal microscopic analysis of the biofilms formed by *P.g.* (×40)
A: Group A; B: Group B (APNPs-treated group, 10 kHz, 1600 ns and 8 kV); C: Group C

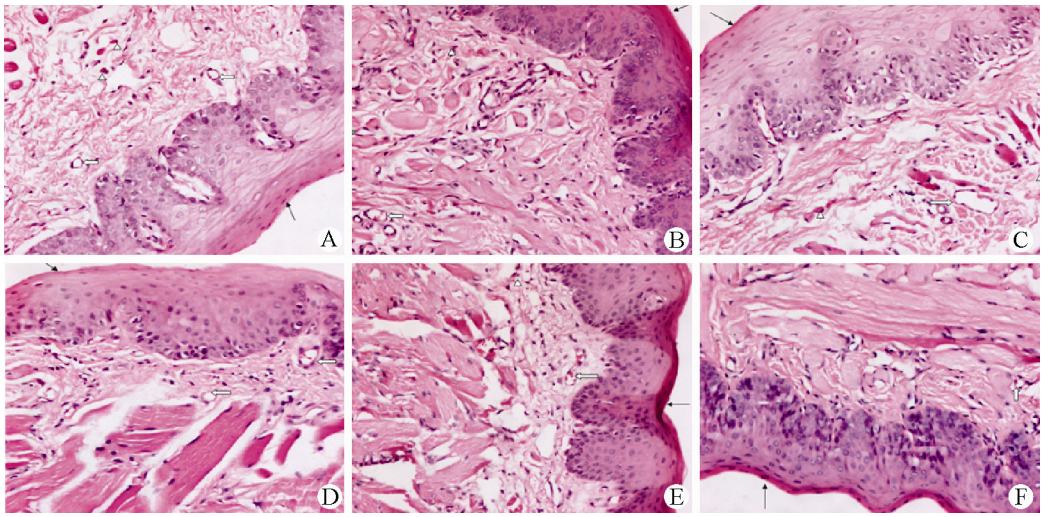

Fig. 5 Histological images of the buccal mucosa of the left side and the mucosa of the ventral surface of the tongue (HE staining, ×20)

A: Normal buccal mucosa; B: Buccal mucosa 1 day after treatment by APNPs; C: Buccal mucosa 5 days after treatment by APNPs; D: Normal mucosa of the ventral surface of the tongue; E: Mucosa of the ventral surface of the tongue 1 day after treatment by APNPs; F: Mucosa of the ventral surface of the tongue 5 days after treatment by APNPs. The inflammatory cells are indicated by open triangle, mucous layer by solid arrow, blood vessel by open arrow.

3 DISCUSSION

Our study demonstrated that APNPs had a conspicuous killing effect on *P.g.* in the biofilms and no significant effect on the histopathology of normal oral mucosa of rabbits. For a novel treatment, the biological safety is a priority that needs to be considered. As indicated in this study, APNPs caused no injury to the normal oral mucosa. There are various microorganisms in the oral cavity. Periodontal diseases and decayed teeth, the commonly occurring diseases in clinical practice, are found to be closely related to pathogenic bacteria. The most important prevention and treatment strategies for these diseases involve local disinfection and complete elimination of infected bacteria. The present study suggested that APNPs may offer such advantages over the traditional methods as high bacterial killing efficiency, time saving and less toxic byproducts produced.

Biofilms are bacterial communities which are enclosed within a matrix of hydrated polymer produced by the bacteria and adhere to a biotic or abiotic surface. Most of bacteria can form biofilms under proper conditions to resist unfavorable environment and the host's defense response. *P.g.* and other oral bacteria can also form biofilms on the teeth, and then induce the periodontal disease. Our results showed that APNPs could kill *P.g.* in the biofilms formed only by *P.g.*. We speculated that APNPs could destroy the biofilms by killing the bacteria in the biofilms and therefore eliminate the pathogenicity of biofilms.

Some studies reported that it took only 4 min to kill *Enterococcus faecalis* by APNPs, and in contrast, these pathogenic bacteria couldn't be killed in root canal therapy with the existing methods[22, 23]. In this study, we choose 10 min as the exposing time for APNPs. And we found that APNPs caused no harm to the oral mucosa in the 10-min treatment. Given the fast self-renewal of oral mucosal epithelial cells (about 7 to 10 days in common), we choose 5 days as the observation time to evaluate the changes of oral mucosal tissue of rabbits after treatment by APNPs. It was found that the histopathology of oral mucosa 5 days after APNPs treatment was presented as normal, on the basis of which, we could further confirm the safety of APNPs to oral mucosa. In addition, the structures of oral mucosa are similar to those of vagina[24], so in the present study, the index used in the vaginal mucous irritation test was employed for evaluation of histopathological changes of oral mucosa.

Until now, the mechanism of steriling effect of APNPs has not been fully understood. Studies found that the particles (such as O, OH), instead of heating and ultraviolet (UV), played an important role in the inactivation of bacteria when He/O_2 was used as working gas[18]. We speculated that the results of our animal experiments could be explained from the following aspects. Firstly, the life time of the atoms is very short, and they work on the bacteria for only several milliseconds and fail to cause injury to the normal tissue; secondly, the mucosa of the animals is one of the natural barrier that can resist stimulations from outside; thirdly, there is a layer of mucus to protect the mucosa; finally, the role of the UV is weak, and it cannot damage the mucosa in such a short period of exposing time.

In conclusion, the present study proved that APNPs can effectively kill the bacteria in the biofilms and cause no harm to oral mucosa of rabbit based on the clinical observations and the pathological examination. However, there are some limitations in our study such as only one strain of pathogenic bacteria used and no involvement of *in vivo* bacterial-killing effect of APNPs. More studies needs to be performed to further validate the effectiveness and safety of APNPs in the treatment of oral diseases in the future.

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REFERENCES

1 Haffajee AD, Socransky S. Microbial etiological agents of destructive periodontal diseases. Periodontol, 2000, 1994,5(1):78-111
2 Mouton C, Hammond PG, Slots J, *et al*. Serum antibodies to oral *Bacteroides asaccharolyticus* (*Bacteroides gingivalis*): relationship to age and periodontal disease. Infect Immun, 1981,31(1):182-192
3 Tanner AC, Milgrom PM, Kent K, *et al*. The microbiota of young children from tooth and tongue samples. J Dent Res, 2002,81(1):53-57
4 Yang EY, Tanner AC, Milgrom P. Periodontal pathogen detection in gingiva/tooth and tongue flora samples from 18- to 48-month-old children and periodontal status of their mothers. Oral Microbiol Immunol, 2002,17(1): 55-59
5 Dzink JL, Tanner AC, Haffajee AD, *et al*. Gram negative species associated with active destructive periodontal lesions. J Clin Periodontol, 1985,12(8):648-659
6 Casarin RC, Ribeiro FV, Sallum AW, *et al*. Root surface defect produced by hand instruments and ultrasonic scaler with different power settings: an *in vitro* study. Braz Dent J, 2009,20(1):58-63
7 Block CM, Mayo JA, Evans GH. Effects of the Nd:YAG dental laser on plasma-sprayed and hydroxyapatite-coated titanium dental implants: surface alteration and attempted sterilization. Int J Oral Maxillofac Implants, 1992,7(4): 441-449
8 Sjogren U, Figdor D, Persson S, *et al*. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J, 1997,30(5):297-306
9 Peters LB, Wesselink PR. Periapical healing of endodontically treated teeth in one and two visits obturated in the presence or absence of detectable microorganisms. Int Endod J, 2002,35(8):660-667
10 Lerouge S, Wertheimer MR, Yahia LH. Plasma sterilization: a review of parameters, mechanisms, and limitations. Plasmas Polym, 2001,6(3):175-188
11 Moisan M, Barbeau J, Moreau S, *et al*. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. Int J Pharm, 2001,226(1–2):1-21
12 Laroussi M. Non-thermal decontamination of biological media by atmospheric pressure plasmas: review, analysis and prospects. IEEE Trans Plasma Sci, 2002,30(4): 1409-1415
13 Sharma A, Pruden A, Zengqi Y, *et al*. Bacterial inactivation in open air by the afterglow plume emitted from a grounded hollow slot electrode. Environ Sci Technol, 2005,39(1):339-344
14 Sladek RE, Stoffels E. Deactivation of *Escherichia coli* by the plasma needle. J Phys D: Appl Phys, 2005,38(11):1716-1721
15 Laroussi M, Mendis DA, Rosenberg M. Plasma interactions with microbes. New J Phys, 2003,5:41.1-41.10
16 Zhou XC, Xiong ZL, Cao YG, *et al*. The antimicrobial activity of an atmospheric-pressure room-temperature plasma in a simulated root-canal model infected with *Enterococcus Faecalis*. IEEE Trans Plasma Sci, 2010,38(12):3370-3374
17 Lu XP, Jiang ZH, Xiong Q, *et al*. An 11 cm long atmospheric pressure cold plasma plume for applications of plasma medicine. Applied Phys Lett, 2008, 92(8):081502-1-081502-2
18 Lu X, Ye T, Cao YG, *et al*. The roles of the various plasma agents in the inactivation of bacteria. J Appl Phys, 2008,104(5):053309-1-053309-5
19 Kieft IE, Kurdi M, Stoffels E. Reattachment and apoptosis after plasma-needle treatment of cultured cells. IEEE Trans Plasma Sci, 2006,34(4):1331-1336
20 Lu X, Jiang Z, Xiong Q, *et al*. A single electrode room-temperature plasma jet device for biomedical applications. Appl Phys Lett, 2008, 92(15):151504-1-151504-3
21 Technical Standard for Disinfection. Ministry of Health of PRC. 2002:143-145
22 Radcliffe CE, Potouridou L, Qureshi R, *et al*. Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms *actinomyces israelii*, *A. naeslundii*, *Candida albicans* and *Enterococcus faecalis*. Int Endod J, 2004,37(7):438-477
23 Ferraria PH, Cai S, Bombana AC, *et al*. Effect of endodontic procedures on enterococci, enteric bacteria and yeasts in primary endodntic infections. Int Endod J, 2005,38(6):372-372
24 Zhou ZZ. Histology and Embryology (Chinese). Fifth edition. Beijing: People's Medical Publishing House, 2001:150-219

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