Plasma treatment effects on destruction and recovery of *Porphyromonas gingivalis* biofilms

Qing Hong¹*, Hongmin Sun²*, Meng Chen³, Shaoping Zhang⁴, Qingsong Yu¹*

¹ Department of Mechanical and Aerospace Engineering, University of Missouri, Columbia, MO, United States of America, ² Department of Internal Medicine, University of Missouri, Columbia, MO, United States of America, ³ Nanova, Inc., Columbia, MO, United States of America, ⁴ Department of Periodontics, College of Dentistry, Iowa University, Iowa City, IA, United States of America

* Current address: Midwestern University, Glendale, AZ, United States of America

sunh@health.missouri.edu (HS); yuq@missouri.edu (QY)

Abstract

The objective of this study was to investigate the treatment effects of non-thermal atmospheric gas plasmas (NTAP) on destruction and the recovery (or re-colonization) of *Porphyromonas gingivalis* (*P. gingivalis*) in biofilms. *P. gingivalis* is a well-known keystone periodontal pathogen strongly associated with periodontal diseases, especially periodontitis. *P. gingivalis* biofilms were formed on stainless steel coupons and treated for 1, 2, and 5 minutes by NTAP of pure argon gas and argon+oxygen gas mixture. MTT assay, colony forming unit (CFU) counting assay and confocal laser scanning microscopy (CLSM) were used to assess the destruction efficiency. In addition, the plasma treated biofilms were re-cultured in the medium supplemented with antibiotics and oxidative stress sources to determine the synergy of the NTAP with other antimicrobial agents. The results showed the plasma treatment could result in 2.7 log unit reduction in bacterial load. The recovered biofilm CFU with NTAP treatment combined with sub minimal inhibition concentration of amoxicillin was 0.33 log units less than the biofilm treated with amoxicillin alone. The recovered biofilm CFU in NTAP groups was about 2.0 log units less than that in the untreated controls under H₂O₂ treatment. There was approximately 1.0 log unit reduction of biofilm CFU in plasma treated biofilm compared with untreated control under paraquat treatment. The plasma treated biofilms exhibited less resistance to amoxicillin and greater susceptibility to hydrogen peroxide (H₂O₂) and paraquat, suggesting that NTAP may enhance biofilm susceptibility to host defense. These *in vitro* findings suggested that NTAP could be a novel and effective treatment method of oral biofilms that cause periodontal diseases.

1. Introduction

Periodontitis is a common oral disease, with a prevalence of 45 to 50% in the world’s population [1]. In US, 42% of adults 30 years or older have periodontitis [2]. It is an inflammatory process in periodontium initiated by dysbiotic plaque bacteria. This extremely common oral disease results in the destruction of periodontal connective tissue and resorption of alveolar
Plasma treatment destructed *Porphyromonas gingivalis* biofilms

Plasma treatment is an innovative treatment modality, with advantages over other antimicrobial methods currently in use. It has the characteristics of an ideal tool, including a high degree of efficacy, fast action, penetrability, lack of toxicity, compatibility with different materials, and cost-effectiveness [23]. NTAP produce a potent cocktail of highly reactive chemical species, including reactive oxygen species (ROS), such as O, O\(^{2-}\), and OH, and reactive nitrogen species (RNS), such as NO and NO\(_2\) [24]. These plasma species are known to exhibit strong oxidative properties and can induce signaling pathways in bacterial cells. For example, oxidation of lipids and proteins that constitute the cell membrane resulted in the loss of their functions, and bacterial cells were found to die in minutes or even seconds in such plasma-induced environment [25]. Lee’s group [26] reported that plasma species penetrated the cell membrane of the microorganisms, leading to cell death in subsequent chemical reactions. Lu and co-workers’ results demonstrated that plasma treatment could effectively kill *P. gingivalis* in biofilms [27] with no harm to oral mucosa of rabbits [19].

Lima et al [28] and Lee et al [29] also demonstrated the effectiveness of plasma disinfection against *P. gingivalis*. In addition, our previous study [30] showed that NTAP as an adjunct therapy further dampened inflammatory response as demonstrated by the decreased protein levels of IL-1\(\beta\) and TNF-\(\alpha\) as well as an enhanced expression of anti-inflammatory marker IL-10 in rat gingival tissue. The reduced bone loss upon NTAP treatment in the rat periodontitis model was partially mediated through the increased microbial killing of periodontitis-associated bacteria including *P. gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia*. Lee et al demonstrated that using NTAP to treat titanium dental implants could prevent bacterial adhesion and biofilm formation on the surface as another useful application for NTAP in clinical application [31]. However, plasma treatment was not able to completely kill *P. gingivalis* bacteria in the biofilms. Moreover, there are few studies of the bacteria regrowth pattern after the plasma treatment..

bone. Approximately 8% of the US adult population has the severe form of this disease that, if left untreated, may lead to tooth loss and is also mechanistically associated with systemic complications [2]. Two major factors contribute to the pathogenesis of periodontitis [3]: first, dysbiotic periodontal pathogens can directly damage the periodontal tissues through the secretion of toxic products; second, the dysregulated host response to periodontal pathogens, which results in release of excess inflammatory mediators (pro-inflammatory cytokines and matrix metalloproteinases), is also involved in the pathology of periodontitis. Specific bacterial species and bacterial complexes occur more frequently in diseased sites, while other bacterial species are associated with periodontal health or stable periodontal lesions [4].

*P. gingivalis* is a well-studied keystone periodontal bacterium that is frequently involved in periodontitis [5,6]. Conventional mechanical debridement (i.e., scaling and root planing) can achieve a temporary reduction of *P. gingivalis* together with other pathogens colonized in the subgingival plaque [6,7]. However, plaque bacteria cannot be effectively removed from the majority of periodontal pockets by this mechanical therapy alone. Infections recur in a significant number of patients in studies by Wasserman [8,9]. Antimicrobial agents may further suppress the periodontal plaque bacteria and increase the benefits of the conventional mechanical treatment. Numerous systemic and locally-delivered antimicrobial agents have been evaluated for the treatment of periodontitis with various degrees of success [3,10–14]. However, a lack of effectiveness of the antibiotics used may be due to development of drug-resistant strains or the dampened metabolic state of the biofilm microflora [15]. To overcome the challenges imposed by the emergence of antibiotic-resistant biofilm bacteria, alternative antimicrobial approaches need to be developed. As a promising alternative approach, plasma treatment using non-thermal atmospheric gas plasmas (NTAP) could effectively kill microbes in localized and topical infections [16–22].

Plasma treatment is an innovative treatment modality, with advantages over other antimicrobial methods currently in use. It has the characteristics of an ideal tool, including a high degree of efficacy, fast action, penetrability, lack of toxicity, compatibility with different materials, and cost-effectiveness [23]. NTAP produce a potent cocktail of highly reactive chemical species, including reactive oxygen species (ROS), such as O, O\(^{2-}\), and OH, and reactive nitrogen species (RNS), such as NO and NO\(_2\) [24]. These plasma species are known to exhibit strong oxidative properties and can induce signaling pathways in bacterial cells. For example, oxidation of lipids and proteins that constitute the cell membrane resulted in the loss of their functions, and bacterial cells were found to die in minutes or even seconds in such plasma-induced environment [25]. Lee’s group [26] reported that plasma species penetrated the cell membrane of the microorganisms, leading to cell death in subsequent chemical reactions. Lu and co-workers’ results demonstrated that plasma treatment could effectively kill *P. gingivalis* in biofilms [27] with no harm to oral mucosa of rabbits [19].

Lima et al [28] and Lee et al [29] also demonstrated the effectiveness of plasma disinfection against *P. gingivalis*. In addition, our previous study [30] showed that NTAP as an adjunct therapy further dampened inflammatory response as demonstrated by the decreased protein levels of IL-1\(\beta\) and TNF-\(\alpha\) as well as an enhanced expression of anti-inflammatory marker IL-10 in rat gingival tissue. The reduced bone loss upon NTAP treatment in the rat periodontitis model was partially mediated through the increased microbial killing of periodontitis-associated bacteria including *P. gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia*. Lee et al demonstrated that using NTAP to treat titanium dental implants could prevent bacterial adhesion and biofilm formation on the surface as another useful application for NTAP in clinical application [31]. However, plasma treatment was not able to completely kill *P. gingivalis* bacteria in the biofilms. Moreover, there are few studies of the bacteria regrowth pattern after the plasma treatment.

fund for the Curators of University of Missouri by Nanova Inc. HS is a co-inventor on patents for methods and compositions for treating bacterial infection: US 8501722, US 9504688, US 9814719, US 10441588, Japan 6293736 and European 2844258. Ivogen Inc. is developing products related to these patents. QY is a cofounder of Nanova Inc. and has financial interest in the company. Nanova has been developing plasma brush device for dental clinical applications, and the related patents include: US 10299887 and US 10631396. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.
In this study, we applied NTAP to P. gingivalis biofilm to examine the antimicrobial effects of plasma treatment and the biofilm recovery after the plasma treatment. The objectives of this study are: 1). To illustrate the mechanism of action of the NTAP on effective destruction of oral biofilms that are pathogenic for periodontal diseases, 2). To investigate the recovery pattern of plasma treated P. gingivalis biofilms under the stress of antibiotics (amoxicillin) and reactive oxygen species (H$_2$O$_2$ and paraquat), simulating host defense against infections.

2. Materials and methods

2.1. Biofilm culture

P. gingivalis (ATCC 33277) was purchased from ATCC (Manassas, VA, USA), and cultured in an anaerobic jar (R685025, Thermo Scientific, Waltham, MA, USA) for biofilm formation, by following the manufacturer’s instruction. Briefly, 1 ml of 5×10$^7$ CFU/ml P. gingivalis suspension was cultured with 1 cm × 1 cm 316L stainless steel coupons in a 24-well plate in an anaerobic incubator. The medium used was Tryptic Soy Broth (TSB) supplemented with Hemin and Vitamin K3, as described in the instructional manual from ATCC. Biofilms were developed for 3 days or 5 days. The medium was replaced at day 2 and day 4.

2.2. Plasma treatments

An NTAP brush used in this study was illustrated in Fig 1A. It was composed of a gas flow controller, a plasma generation chamber, and a direct current (DC) power supply to produce plasma. The details of the plasma brush have been reported previously [32,33]. Briefly, the argon and oxygen gas flow rates were controlled by a MKS mass flow controller (MKS Instrument Andover, MA, USA) and input into the plasma generation chamber containing two needle-shaped electrodes. A DC power supply (Spellman SL60) (Spellman High Voltage Electronics Corporation, Hauppauge, NY, USA) was used to ignite and sustain the plasma. The plasma brush was operated under 6 mA with feeding gas of either pure argon at 3000 standard cubic centimeter per minute (sccm) or argon/oxygen mixture of 3000 sccm argon + 30 sccm oxygen. These parameters had been optimized to achieve stable plasma with gas temperature of 37˚C, which is the normal human body temperature.

Well-developed biofilms were rinsed 3 times with phosphate buffered saline (PBS) to remove the unattached bacteria. Coupons with biofilms on the surfaces were transferred onto the lid of a 24-well plate. An NTAP brush was applied to the biofilms by scanning for a preset time of 1, 2, and 5 minutes, respectively. The procedures were illustrated in Fig 1B.

https://doi.org/10.1371/journal.pone.0274523.g001

Fig 1. Experiment illustrations. A) sketch of the plasma brush, B) Experimental flow chart of the direct plasma destruction on biofilms, C) Experimental flow chart of the biofilm recovery after plasma treatment.
2.3. MTT assay and CFU assay

MTT assay was used to assess the destruction efficiency of plasma against *P. gingivalis* biofilms. The plasma treated biofilm was stained with 0.5 ml MTT solution for 3 hours. MTT solution was removed and 0.4 ml of mixture of DMSO and alcohol (1:1) was added into the well to dissolve the stain. One hundred μl stain liquid of each sample was collected into a 96-well plate to measure optical density (OD) value at 575nm. The bacterial survival percentage was normalized with untreated control sample as 100%.

CFUs of the survival bacteria in the biofilms after plasma treatment were quantified. Each sample after treatment was transferred into a 5 ml centrifuge tube with 2 ml PBS. The tubes were then vortexed for 15 seconds and ultra-sonicated for 5 cycles to detach the bacteria from the coupons. The CFUs in the bacterial suspensions were counted by series dilution on agar plates. After 2 days of incubation, the CFUs were counted to assess the survived bacteria.

2.4. CLSM assay

Three-day old biofilms were stained with Live/Dead staining kit (L13152, Invitrogen, Waltham, MA, USA) by following the manufacturer’s instruction, and observed by a Carl Zeiss LSM 510 confocal laser scanning microscope (CLSM). Random locations of the biofilms were scanned with two channels of laser with an interval of 1 μm.

2.5. Biofilm recovery

Three-day biofilms were treated with plasma and gas blow. Afterwards, the treated biofilms were re-cultured in fresh TSB medium and incubated at 37˚C for 2 days. At the same time, biofilms that did not receive plasma treatment were cultured for 2 more days as controls. The recovered biofilms were assessed by MTT assay and CFU counting. The procedures were illustrated in Fig 1C.

2.6. Susceptibility to the antibiotic

During the recovery period, different concentrations of amoxicillin were added in the culture medium at the beginning of the recovery. After 2 days of recovery, MTT assay and CFU counting were performed to quantitatively assess the biofilms.

2.7. Susceptibility to oxidative stresses

Hydrogen peroxide and paraquat were used as the sources of oxidative stresses. During the recovery period, various concentrations of hydrogen peroxide and paraquat were added in the culture medium at the beginning of the recovery. After 2 days of recovery, MTT assay and CFU counting were performed to quantitatively assess the biofilms.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was performed on all data by using the SPSS statistics software (IBM, Armonk, NY). Tukey’s honestly significant difference post hoc test was performed to compare each group. If p < 0.05 is detected, these factors were considered to have a statistically significant effect at 95% confidence level.
3. Results

3.1. Plasma destruction on *Porphyromonas gingivalis* biofilms

NTAP’s effects on *P. gingivalis* biofilms on stainless steel were assessed. MTT assay was used to assess the destruction efficiency of plasma against *P. gingivalis* biofilms. Fig 2 presented the change of survival percentages of 3-day *P. gingivalis* biofilms with plasma treatment times. Plasma treatment exhibited high efficiency of destruction against 3-day *P. gingivalis* biofilms. Argon plasma treatment resulted in 76.97 ± 1.21%, 75.09 ± 1.80% and 71.95 ± 4.41% bacterial load reduction with 1-, 2- and 5-min treatment time, respectively (p < 0.05). Addition of 1 vol.% oxygen in the argon plasma improved the destruction efficiency, leading to 79.62 ± 1.57%, 80.02 ± 1.78% and 83.38 ± 2.25% bacterial load reduction with 1-, 2- and 5-min treatment time, respectively (p < 0.05). CFU counting assay (Fig 3) was consistent with the MTT results. A reduction of 2.3 log units of CFU (p < 0.05) was achieved by the argon plasma treatment, while addition of 1 vol.% oxygen led to a further reduction of 0.4 log unit CFU (p < 0.05).

Fig 4 shows MTT assay results of the plasma treatment effects on the 5-day *P. gingivalis* biofilms with different treatment times using pure argon plasmas and 1 vol.% oxygen addition into argon plasmas. Argon plasma treatment resulted in 40.24 ± 6.56%, 46.45 ± 4.78%, and 36.36 ± 8.91% bacterial load reduction with 1-, 2- and 5-min treatment time, respectively. Addition of 1 vol.% oxygen in argon plasmas further improved the disinfection, in agreement with results of treatment of 3-day old *P. gingivalis* biofilms. Addition of oxygen in the plasma reduced the bacterial load by 58.05 ± 5.19%, 75.32 ± 5.49% and 81.93 ± 2.58% with 1-, 2- and 5-min treatment time, respectively. Fig 5 compared the plasma treatment against 3-day biofilms and 5-day biofilms. Plasma treatment with 1% oxygen addition resulted in less bacteria CFU (p < 0.05) for 3-day biofilms (2.69 log units of CFU) than that for 5-day biofilms (4.07 log units of CFU). Therefore, only 3-day biofilms were used in the biofilm recovery studies.

The untreated and argon+1% oxygen plasma treated 3-day old *P. gingivalis* biofilms were examined using CLSM, with red color representing dead bacterial cells and green color representing living bacterial cells (Fig 6). As seen in the CLSM images, most of the cells in the untreated control group were alive. Dead bacterial cells appeared after 1-min plasma treatment.
treatment, and a majority of the bacterial cells were dead after 5-min plasma treatment, indicating that plasma destruction of *P. gingivalis* biofilms was time dependent, being consistent with the results from MTT assay and CFU counting assay. Meanwhile, the plasma was able to penetrate into the biofilm structure to cause the biofilm destruction, as demonstrated in the 3D structure figures of the biofilms showing widespread distribution of dead cells (Fig 6E and 6F).

### 3.2. Recovery performance of plasma treated *P. gingivalis* biofilms

The biofilm bacterial loads after 2 days of recovery from untreated and argon+1% oxygen plasma treated 3-day *P. gingivalis* biofilms were examined (Fig 7). There was certain but not significant reduction of bacterial load in plasma treated biofilms as compared to that in the untreated biofilms. MTT assay results showed that, after 2 days of recovery, the bacterial load in the plasma treated group was 6% less than that in the untreated control group. The biofilm CFU of the plasma treated group was 1.1 log units less than the CFU of the untreated control group. However, the difference between the control and plasma treated group was not statistically significant after 2 days of recovery.

Amoxicillin was applied as an antibiotic to prevent *P. gingivalis* biofilm recovery (Fig 8). Reported *in vitro* studies have demonstrated that minimal inhibitory concentration (MIC) of amoxicillin against planktonic *P. gingivalis* is around or less than 0.125 (1/8) μg/ml while bacteria in biofilms are more resistant to the antibiotic [14,34]. After 2 days of recovery under the stress of antibiotic, the bacterial survival percentage of argon+1% oxygen plasma treated *P. gingivalis* biofilms was 21.45% and 14.5% less (p<0.05) than the untreated biofilm controls for the groups with 1/32 and 1/16 μg/mL amoxicillin, respectively, in the recovery medium (Fig 8A). CFU counting results (Fig 8B) showed the same trend. Adding 1/16 μg/mL amoxicillin in the
Fig 4. MTT assay results of plasma destruction of 5-day *Porphyromonas gingivalis* biofilms. * denotes statistically significant difference compared with the control group with $p < 0.05$ according to ANOVA Tukey test. # denotes statistically significant difference between pure argon plasma and argon+1% oxygen plasma treatments with $p < 0.05$ according to ANOVA Tukey test. denotes the statistically significant differences among different treatment times of argon+1% oxygen plasma with $p < 0.05$ according to ANOVA Tukey test.

https://doi.org/10.1371/journal.pone.0274523.g004

Fig 5. CFU counting assay results of argon+1% oxygen plasma destruction of 3-day and 5-day *P. gingivalis* biofilms. * denotes the statistically significant difference compared with the untreated control groups with $p < 0.05$. # denotes the statistically significant difference between 3-day and 5-day biofilms with $p < 0.05$ according to ANOVA Tukey test. denotes the statistically significant differences among different treatment times of 3-day biofilms with $p < 0.05$ according to ANOVA Tukey test. denotes the statistically significant differences among different treatment times of 5-day biofilms with $p < 0.05$ according to ANOVA Tukey test.

https://doi.org/10.1371/journal.pone.0274523.g005
recovery medium, the biofilm CFU with argon+1% oxygen plasma treatment was 0.33 log units (p < 0.05) less than the biofilm CFU without plasma treatment.

H₂O₂ was used as an oxidative stress inducer. The H₂O₂ responses by the untreated and plasma treated 3-day P. gingivalis biofilms were assessed (Fig 9). The H₂O₂ effects on P. gingivalis biofilms were concentration dependent. In comparison with untreated controls, plasma treated P. gingivalis biofilms were much more sensitive to H₂O₂. Even at a very low concentration of 0.002 wt% H₂O₂, 92.4±0.22% reduction in bacterial survival percentage was obtained. It should be pointed out that the similar inhibition effect for the untreated controls required a 4 time higher H₂O₂ concentration, i.e. 0.008 wt% H₂O₂, as shown in both the MTT assays (Fig 9A), and the CFU counting assay (Fig 9B). The recovered biofilm CFUs in the plasma treated groups were 1.91 and 2.00 log units less than (p < 0.05) that in the untreated controls under H₂O₂ concentrations of 0.002 wt% and 0.004 wt%, respectively. When H₂O₂ concentration was higher than 0.004 wt%, all bacteria were dead in both plasma treated groups and the untreated controls.

Paraquat was used as another oxidative stress inducer. The paraquat responses by the untreated and plasma treated 3-day P. gingivalis biofilms were assessed (Fig 10). Plasma treated 3-day P. gingivalis biofilms were also more sensitive to paraquat treatment than the untreated control. The survival percentage of the plasma treated P. gingivalis biofilms was only 43.40 ±1.80%, which was significantly lower (p < 0.05) than the 90.79±6.67% survival percentage observed with the untreated controls when treated with 12.5 mM paraquat according to MTT assay (Fig 10A). Consistent results were also obtained with the CFU counting assay (Fig 10B). There was 1.5 log unit reduction (p < 0.05) of biofilm CFU in plasma treated P. gingivalis biofilms while only 0.57 log unit reduction in the untreated control when treated with 12.5 mM paraquat (Fig 10B). Compared with the control groups without plasma treatments, the argon +1% oxygen plasma treatments were able to further reduce the bacterial load by 0.38 and 0.56 log units (p < 0.05) when treated with 25 mM and 50 mM paraquat, respectively, in the recovery medium.
4. Discussion

Our recent research has shown that NTAP can rapidly and effectively disinfect not only planktonic bacteria of *Escherichia coli*, *Micrococcus luteus*, *Streptococcus mutans* and *Lactobacillus acidophilus* on various surfaces [20,35,36], but also bacteria in caries related biofilms [32,33] and fungus in candidiasis related biofilms as well [37]. The *in vitro* data collected in this study demonstrated that NTAP could be effective in destructing and reducing *P. gingivalis* biofilm reformation. MTT assay results showed that the bacterial load reduction in *P. gingivalis* biofilms was greater than 80% after plasma treatment by the argon+1% oxygen plasma for 5 minutes. CFU counting assay results also showed a 2.7 log unit reduction. These results indicated that NTAP could destroy the biofilms by killing the bacteria in *P. gingivalis* biofilms and therefore mitigate the pathogenicity of the biofilms.

In this study, 316L stainless steel, a medical grade of metallic alloy, was used as the surface for biofilm formation. 316L has been used in dental implants due to its good biocompatibility, excellent corrosion resistance, and high mechanical strength. Specifically, its applications in dentistry include: temporary crowns, arch wires, and brackets. Stainless steel coupons have been used by other research group for *P. gingivalis* biofilm growth [38]. Stainless steel was also
utilized for studying adhesion of *S. aureus* to its surface for dental application [39]. Additional biomaterials used for making dental implants are titanium (Ti) and its alloy, Ti6Al4V. We are currently testing the plasma effect on biofilms formed on the titanium surface.

Plasma destruction efficiency of *P. gingivalis* biofilms depended on the biofilm structure and exposure time. Plasma treatment was less effective in microbial killing in 5-day *P. gingivalis* biofilms than that in 3-day biofilms, possibly because the biofilms were more mature after 5-day culture than after 3-day culture. There were more bacteria in the 5-day biofilms, and the mature biofilms provided stronger protection for the biofilm bacteria from the plasma treatment. The increase of plasma exposure time improved the killing of the biofilm bacteria, as it took time for plasma species to penetrate into the biofilm to destruct the bacteria in the biofilms.

![Graph](https://doi.org/10.1371/journal.pone.0274523.g008)

**Fig 8.** Antibiotic susceptibility results from (A) MTT assay and (B) CFU counting assay for untreated and 1-min argon+1% oxygen plasma treated 3-day *P. gingivalis* biofilms. * denotes the statistically significant difference (p < 0.05) compared with the control groups without amoxicillin application (0 μg/ml). # denotes the statistically significant difference (p < 0.05) between untreated controls and plasma treated groups.
In terms of plasma gas source, the addition of oxygen in argon plasma could improve the plasma destruction efficiencies on *Porphyromonas gingivalis* biofilms. This can be attributed to the interaction of reactive oxygen species (ROS) in the plasmas with the bacteria. Production of more ROS with oxygen addition in the argon plasmas could lead to improvement of the destruction efficiency because ROS is one of the main plasma species causing disinfection. The ROS such as O\(^{-}\) and O\(_2^\cdot\) radicals in the plasmas can penetrate and destroy the microorganism structures through oxidation processes [40]. As reported by Lu et al. [41], the disinfection area enlarged significantly when the operating gas of He/H\(_2\) was replaced by He/O\(_2\).

Longer plasma treatment time was required to inactivate *P. gingivalis* biofilms, which could be due to the morphological structure of *P. gingivalis*. The ATCC 33277 strain of *P. gingivalis*
used in the study is not capsulated standard strain. As a gram-negative anaerobic bacterium, *P. gingivalis* has an outer membrane, a peptidoglycan layer, and a cytoplasmic membrane [42,43]. The unique lipopolysaccharide structure of *P. gingivalis* might contribute its resistance to antimicrobial peptides [44]. Mahasneh et al also reported that *P. gingivalis* was less susceptible to plasma treatment than other bacteria [45].

Similar to the conventional mechanical treatment, it was very difficult to completely kill the bacteria in *P. gingivalis* biofilms with plasma treatment even though it exhibited high microbial killing efficiency against *P. gingivalis* biofilms. The recolonization of the plasma treated *P. gingivalis* biofilms was critical for clinical application of NTAP to thwart reoccurrence of the biofilm infections [46]. Bacteria regrowth will make eradication of biofilm infection difficult. In
clinical practice, antimicrobial agents are sometimes used as an adjunct therapy to mechanical debridement. For example, amoxicillin is prescribed to patients with the molar-incisor pattern (formerly "aggressive periodontitis") in conjunction with the scaling and root planing. Therefore, it is clinically relevant to investigate the recovery pattern of plasma treated \textit{P. gingivalis} biofilms under the stress of antibiotics (amoxicillin) \cite{34} and reactive oxygen species ($\text{H}_2\text{O}_2$ and paraquat), which simulate the host immune response in controlling oral biofilms \cite{47}.

Antibiotics, typically amoxicillin, are sometime prescribed as a combinatory antimicrobial therapy in conjunction with mechanical debridement or scaling and root planing under certain circumstances such as periodontitis with molar-incisor pattern or disease with refractory nature and resistant to treatment. Amoxicillin was tested in this study because it is the first line of the most frequently used antibiotic in clinic when antibiotics are regarded as necessary as an adjunct therapy. Previous studies on the minimal inhibitory concentration (MIC) of amoxicillin against 50\% \textit{P. gingivalis} clinical isolates in planktonic form is about 0.063 (1/16) $\mu$g/ml to 0.125 (1/8) $\mu$g/ml \cite{14,34}. Different sub-MIC dosages of amoxicillin were tested in this study because such titrations would maximally expose the adjunctive effect of NTAP treatment in a titratable range. The results obtained in this study showed that plasma treated \textit{P. gingivalis} biofilms were more susceptible to amoxicillin and their survival percentage was significantly decreased compared with that of the untreated biofilms. This finding indicates that the plasma treatment could increase the susceptibility of \textit{P. gingivalis} biofilms to antibiotic treatment. In clinical practices, some patients with advanced periodontal diseases do not respond to conventional mechanical periodontal treatment, including surgical procedures, but need supplemental antibiotic treatments. Periodontal diseases are often associated with the presence of specific periodontal pathogens such as \textit{P. gingivalis}, an exogenous pathogen that needs to be eradicated, if necessary by the administration of antibiotics \cite{48}. The clinical outcome of antibiotic therapy is, however, not always satisfactory \cite{49}. The most likely explanation of the treatment failure is that the subgingival bacteria present in biofilms are more resistant to antimicrobial treatment than planktonic cultures used for antimicrobial susceptibility testing \cite{50,51}. The results obtained in this study indicate that, as a supplemental to the antibiotic therapies, plasma treatment has a significant benefit to improve clinical outcome.

In periodontal inflammation, polymorphonuclear leukocytes (PMN) served as the initial host defense against periodontal pathogens including \textit{P. gingivalis}. After stimulation by bacterial antigens and during phagocytosis, PMN produces ROS such as hydrogen peroxide, single oxygen and hydroxyl radicals. ROS are the main elements for bactericidal activity of the host defense \cite{52}. The antioxidant defense mechanisms of several bacteria were investigated by adding ROS-generating agents to the culture medium, which experimentally simulated the oxidative stress in host defense \cite{47}. Hydrogen peroxide ($\text{H}_2\text{O}_2$) and paraquat are two widely used sources to provide ROS for \textit{in vitro} experiments \cite{53–58}. Hydrogen peroxide can produce singlet oxygen, superoxide radicals and hydroxy radical to damage the bacterial components such as enzymes, membrane constituents and DNA \cite{59}. Paraquat can catalyze the formation of superoxide free radicals and cause toxic reactions such as peroxidation of polyunsaturated lipid, depolymerization of hyaluronic acid, inactivation of proteins and damage to DNA \cite{60}.

One of our previous studies \cite{33} demonstrated plasma treated caries-related biofilms displayed significantly lower tolerance to the $\text{H}_2\text{O}_2$ and paraquat compared to untreated biofilms. In this study, these two ROS source reagents were used to assess the response of plasma treated \textit{P. gingivalis} biofilms to oxidative stress. Our results demonstrated that plasma treated \textit{P. gingivalis} biofilms exhibited significantly lower tolerance to $\text{H}_2\text{O}_2$ and paraquat, compared with untreated biofilms. The biofilms were more resistant to the attacking and killing by the host immune systems than planktonic bacteria, contributing to the enhanced virulence of biofilm infections \cite{61,62}. Generation of ROS by PMN is one of the important steps to mediate the
defense against bacterial pathogens. The results suggest that the plasma treated *Porphyromonas gingivalis* biofilms could be better controlled by the host immune system than untreated biofilms. Although the *in vitro* generated oxidation environment by H$_2$O$_2$ and paraquat might not accurately recapitulate the *in vivo* generation of ROS by PMN in host defense system, our previous study showed that NTAP could not only reduce periodontitis-induced alveolar bone loss in rats but also reduce the periodontitis-associated bacteria burden, supporting our hypothesis that NTAP enhances host defense against periodontitis-associated bacterial infections [30].

5. Conclusion

In conclusion, plasma treatment using NTAP with both pure argon gas and its mixture with 1 vol.% oxygen demonstrated destruction efficacy on *Porphyromonas gingivalis* biofilms. The addition of oxygen in argon plasmas further improved the plasma destruction efficiency. Plasma destruction efficiency on *P. gingivalis* biofilms also depended on the biofilm structure and treatment time. More importantly, the plasma treatment could significantly reduce the resistance of *P. gingivalis* biofilms to antibiotics and increase the susceptibility of the biofilms to oxidative stresses, suggesting plasma treatment could enhance host control of biofilm infection. Even though the plasma treatment could not completely prevent biofilm recovery, the recovery of *P. gingivalis* biofilms could be better controlled when the plasma treatment is applied in combination with other therapies. Future studies are still needed to assess NATP’s microbial killing efficiency of multi-species oral biofilms on other biomaterial substrates and the effect on inflammatory response in cell culture and the tissue repair after plasma treatment in animal periodontitis models. Plasma treatment using NTAP could have great potential to become an effective treatment method to disinfect and control pathogenic microorganisms in dental patients with periodontal diseases.

Acknowledgments

We would like to Dr. John E. Jones for proof reading the manuscript.

Author Contributions

Conceptualization: Hongmin Sun, Meng Chen, Qingsong Yu.

Formal analysis: Qing Hong, Hongmin Sun, Meng Chen, Qingsong Yu.

Funding acquisition: Meng Chen, Qingsong Yu.

Investigation: Qing Hong.

Methodology: Qing Hong, Hongmin Sun, Meng Chen, Qingsong Yu.

Project administration: Hongmin Sun, Meng Chen, Qingsong Yu.

Supervision: Hongmin Sun, Qingsong Yu.

Writing – original draft: Qing Hong, Hongmin Sun, Meng Chen, Shaoping Zhang, Qingsong Yu.

Writing – review & editing: Qing Hong, Hongmin Sun, Meng Chen, Shaoping Zhang, Qingsong Yu.

References

1. Sanz M, Marco Del Castillo A, Jepsen S, Gonzalez-Juanatey JR, D’Alito F, Bouchard P, et al. Periodontitis and cardiovascular diseases: Consensus report. J of clin peri. 2020; 47(3):268–88.
2. Eke PI, Thornton-Evans GO, Wei L, Borgnakke WS, Dye BA, Genco RJ. Periodontitis in US Adults: National Health and Nutrition Examination Survey 2009–2014. Journal of the American Dental Association (1939). 2018; 149(7):576–88.e6. https://doi.org/10.1016/j.adaj.2018.04.023 PMID: 29957185
3. Bodet C, Piché M, Chandad F, Grenier D. Inhibition of periodontopathogen-derived proteolytic enzymes by a high-molecular-weight fraction isolated from cranberry. Journal of Antimicrobial Chemotherapy. 2006; 57(4):685–90. https://doi.org/10.1093/jac/dkl031 PMID: 16473919
4. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998; 25(2):134–44. https://doi.org/10.1111/j.1600-051x.1998.tb02419.x PMID: 9495612
5. Slots J, Ting M. Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in human periodontal disease: occurrence and treatment. Periodontology 2000. 1999; 20(1):82–121. https://doi.org/10.1111/j.1600-0757.1999.tb00159.x PMID: 10522224
6. Cutler CW, Kalmar JR, Genco CA. Pathogenic strategies of the oral anaerobe, Porphyromonas gingivalis. Trends Microbiol. 1995; 3(2):45–51. https://doi.org/10.1016/s0966-842x(00)88874-5 PMID: 7728384
7. Lamont RJ, Jenkinson HF. Subgingival colonization by Porphyromonas gingivalis. Oral Microbiology and Immunology. 2000; 15(6):341–9. https://doi.org/10.1034/j.1399-302x.2000.150601.x PMID: 11154429
8. Wasserman B, Hirschfeld L. The relationship of initial clinical parameters to the long-term response in 112 cases of periodontal disease. J Clin Periodontal. 1988; 15(1):38–42. https://doi.org/10.1111/j.1600-051x.1988.tb01552.x PMID: 342224
9. Newman MG, Takei H, Klokkevold PR, Carranza FA. Carranza’s clinical periodontology: Elsevier health sciences; 2011.
10. Bavington C, Page C. Stopping bacterial adhesion: a novel approach to treating infections. Respiration; international review of thoracic diseases. 2005; 72(4):335–44. https://doi.org/10.1159/000086243 PMID: 16088272
11. Pajukanta R. In vitro antimicrobial susceptibility of Porphyromonas gingivalis to azithromycin, a novel macrolide. Oral Microbiology and Immunology, 1993; 8(5):325–6. https://doi.org/10.1111/j.1399-302x.1993.tb00583.x PMID: 8265209
12. Wright TL, Ellen RP, Lacroix JM, Speight P, Wilson M. In Vivo Killing of Porphyromonas gingivalis by Toluidine Blue-Mediated Photosensitization in an Animal Model. Anti Agen and Chem. 2003; 47(3):932–40. https://doi.org/10.1128/AAC.47.3.932-940.2003 PMID: 12604524
13. Larsen T. Susceptibility of Porphyromonas gingivalis biofilms to amoxicillin, doxycycline and metronidazole. Oral Microbiol Immunol. 2002; 17(5):267–71. https://doi.org/10.1034/j.1399-302x.2002.170501.x PMID: 12354206
14. Walker CB. The acquisition of antibiotic resistance in the periodontal microflora. Periodontology 2000. 1996; 10:79–88. https://doi.org/10.1111/j.1600-0757.1996.tb00069.x PMID: 9567938
15. Moreau M, Orange N, Feuilloley M. Non-thermal plasma technologies: new tools for bio-decontamination. Biotechnology advances. 2008; 26(6):610–7. https://doi.org/10.1016/j.biotechadv.2008.08.001 PMID: 18775485
16. Taghizadeh L, Brackman G, Nikiforov AY, Coenye T, Leys C. Antibiofilm effect of argon plasma jet. Proceeding s of ISPC international symposium on plasma chemistry, Bochum 2009 Jul (pp. 26–31).
17. De Geyter N, Morent R. Nonthermal plasma sterilization of living and nonliving surfaces. Annual Review of Biomedical Engineering. 2012; 14:255–74. https://doi.org/10.1146/annurev-bioeng-071811-150110 PMID: 22559318
18. Liu D, Xiong Z, Du T, Zhou X, Cao Y, Lu X. Bacterial-killing effect of atmospheric pressure non-equilibrium plasma jet and oral mucosa response. Journal of Huazhong University of Science and Technology [Medical Sciences]. 2011; 31:852–6. https://doi.org/10.1007/s11596-011-0690-y PMID: 22173512
19. Yu Q, Huang C, Hsieh FH, Huff H, Duan Y. Bacterial inactivation using a low-temperature atmospheric plasma brush sustained with argon gas. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2007; 80(1):211–9.
20. Moisant M, Barbeau J, Crevier M-C, Pelletier J, Philip N, Saoudi B. Plasma sterilization. Methods and mechanisms. Pure and Applied Chemistry. 2002; 74(3):349–58.
21. Koban I, Geisel MH, Holftruter B, Jablonowski L, #xfc, bner N-O, et al. Synergistic Effects of Nonthermal Plasma and Disinfecting Agents against Dental Biofilms in Vitro. ISRN Dentistry. 2013; 2013:10. https://doi.org/10.1155/2013/573262 PMID: 24159388
23. Boscaroli M, Moreira A, Mansano R, Kikuchi I, Pinto T. Sterilization by pure oxygen plasma and by oxygen–hydrogen peroxide plasma: An efficacy study. International journal of pharmaceutics. 2008; 353(1):170–5.

24. Laroussi M. Low-Temperature Plasma Jet for Biomedical Applications: A Review. Plasma Science, IEEE Transactions on. 2015; 43(3):703–12.

25. Laroussi M. Low Temperature Plasma-Based Sterilization: Overview and State-of-the-Art. Plas Proc and Pol. 2005; 2(5):391–400.

26. Lee K, Paek KH, Ju WT, Lee Y. Sterilization of bacteria, yeast, and bacterial endospores by atmospheric-pressure cold plasma using helium and oxygen. Journal of microbiology (Seoul, Korea). 2006; 44(3):269–75.

27. Xiong Z, Du T, Lu X, Cao Y, Pan Y. How deep can plasma penetrate into a biofilm? Appl Phys Lett. 2011; 98(22):221503–3.

28. Lima GdMG, Borges AC, Nishime TMC, Santana-Melo GdF, Kostov KG, Mayer MPA, et al. Cold Atmospheric Plasma Jet as a Possible Adjuvant Therapy for Periodontal Disease. Molecules (Basel, Switzerland). 2021; 26(18):5590. https://doi.org/10.3390/molecules26185590 PMID: 34577061

29. Lee J-Y, Kim K-H, Park S-Y, Yoon S-Y, Kim G-H, Lee Y-M, et al. The bactericidal effect of a non-thermal atmospheric pressure plasma treatment of titanium surfaces according to the bacterial wall structure. Scientific Reports. 2019; 9(1):1938. https://doi.org/10.1038/s41598-019-39414-9 PMID: 30760871

30. Zhang Y, Xiong Y, Xie P, Ao X, Zheng Z, Dong X, et al. Non-thermal plasma reduces periodontitis-induced alveolar bone loss in rats. Biochemical and Biophysical Research Communications. 2018; 503(3):2040–6. https://doi.org/10.1016/j.bbrc.2018.07.154 PMID: 30086885

31. Lee M-J, Kwon J-S, Jiang HB, Choi EH, Park G, Kim K-M. The antibacterial effect of a non-thermal atmospheric pressure plasma treatment of titanium surfaces according to the bacterial wall structure. Scientific Reports. 2019; 9(1):1938. https://doi.org/10.1038/s41598-019-39414-9 PMID: 30760871

32. Hong Q, Dong X, Chen M, Xu Y, Sun H, Hong L, et al. Disinfection of Streptococcus mutans biofilms on sandblasted and acid-etched titanium discs. Journal of periodontal & implant science. 2019; 49(5):319–29. https://doi.org/10.5051/jpis.2019.49.5.319 PMID: 31681489

33. Zhang Y, Xiong Y, Xie P, Ao X, Zheng Z, Dong X, et al. Non-thermal plasma reduces periodontitis-induced alveolar bone loss in rats. Biochemical and Biophysical Research Communications. 2018; 503(3):2040–6. https://doi.org/10.1016/j.bbrc.2018.07.154 PMID: 30086885

34. Yu Q, Hong Q, Dong X, Chen M, Sun H, Hong L, et al. In vitro and in vivo study of plasma treatment effects on oral biofilms. Journal of Oral Microbiology. 2019; 11(1):1603524. https://doi.org/10.1080/20002297.2019.1603524 PMID: 31069019

35. Ardila CM, López MA, Guzmán IC. High resistance against clindamycin, metronidazole and amoxicillin in Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans isolates of periodontal disease. Med Oral Patol Oral Cir Bucal. 2010; 15(6):e947–e51. PMID: 20383102

36. Huang C, Yu Q, Hsieh F-h, Duan Y. Bacterial Deactivation Using a Low Temperature Argon Atmospheric Plasma Brush with Oxygen Addition. Plas Proc and Pol. 2007; 4(1):77–87.

37. Blumhagen A, Singh P, Mustapha A, Chen M, Wang Y, Yu Q. Plasma Deactivation of Oral Bacteria Seeded on Hydroxyapatite Disks as Tooth Enamel Analogue. American journal of dentistry. 2014; 27(2):84–90. PMID: 25000666

38. Yu Q, Hong Q, Dong X, Chen M, Sun H, Hong L, et al. Plasma Treatment Effects on Oral Candida albicans Biofilms. Dental Oral Biology and Craniofacial Research. 2021:1–7.

39. El Aouame A, El Quars F, Bentahar Z, Zerouali K, Sidqui M. In Vitro Evaluation of Bacterial Adhesion to Dental and Stainless-Steel Surfaces. Open Journal of Medical Microbiology. 2021; 11(3):176–97.

40. Huang C, Yu Q, Hsieh F-h, Duan Y. Bacterial Deactivation Using a Low Temperature Argon Atmospheric Plasma Brush with Oxygen Addition. Plas Proc and Pol. 2007; 4(1):77–87.

41. Lu X, Ye T, Cao Y, Sun Z, Xiong Q, Tang Z, et al. The roles of the various plasma agents in the inactivation of bacteria. Journal of Applied Physics. 2008; 104(5):053309–5.

42. Beveridge TJ. Structures of gram-negative cell walls and their derived membrane vesicles. Journal of bacteriology. 1999; 181(16):4725–33. https://doi.org/10.1128/JB.181.16.4725-4733.1999 PMID: 10438737

43. Gan L, Chen S, Jensen GJ. Molecular organization of Gram-negative peptidoglycan. Proceedings of the National Academy of Sciences. 2008; 105(48):18953–7. https://doi.org/10.1073/pnas.080035105 PMID: 19033194

44. Bachrach G, Altman H, Kolenbrander PE, Chalmers NI, Gabai-Gutner M, Mor A, et al. Resistance of Porphyromonas gingivalis ATCC 33277 to direct killing by antimicrobial peptides is protease
45. Mahasneh A, Darby M, Tolle SL, Hynes W, Laroussi M, Karakas E. Inactivation of Porphyromonas gingivalis by low-temperature atmospheric pressure plasma. Plasma Medicine. 2011; 1(3–4).

46. Rams TE, Listgarten MA, Slots J. Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis subgingival presence, species-specific serum immunoglobulin G antibody levels, and periodontitis disease recurrence. Journal of Periodontal Research. 2006; 41(3):228–34. https://doi.org/10.1111/j.1600-0765.2005.00860.x PMID: 16677293

47. Oliveira MVd, Oliveira AcFd, Shida CS, Oliveira RCd, Nunes LR. Gene expression modulation by paraquat-induced oxidative stress conditions in Paracoccidioides brasiliensis. Fungal Genetics and Biology. 2013; 60:101–9. https://doi.org/10.1016/j.fgb.2013.05.004 PMID: 23711636

48. Winkelhöfl AJV, Rams TE, Slots J. Systemic antibiotic therapy in periodontics. Periodontology 2000. 1996; 10(1):45–78. https://doi.org/10.1111/j.1600-0757.1996.tb0068.x PMID: 9567937

49. Bollen CM, Quirynen M. Microbiological response to mechanical treatment in combination with adjunctive therapy. A review of the literature. J Periodontol. 1996; 67(11):1143–58. https://doi.org/10.1902/jop.1996.67.11.1143 PMID: 8959563

50. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999; 284(5418):1318–22. https://doi.org/10.1126/science.284.5418.1318 PMID: 10334980

51. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. Periodontology 2000. 1997; 14(1):12–32. https://doi.org/10.1111/j.1600-0757.1997.tb00190.x PMID: 9567964

52. Leto TL, Geiszt M. Role of NADPH oxidases in host defense. Antioxidants & redox signaling. 2006; 8(9–10):1549–61. https://doi.org/10.1089/ars.2006.8.1549 PMID: 16987010

53. Hyslop PA, Hinshaw DB, Scrautfatter IU, Cochrane CG, Kunz S, Vosbeck K. Hydrogen peroxide as a potent bacteriostatic antibiotic: Implications for host defense. Free Radical Biology and Medicine. 1995; 19(1):31–7. https://doi.org/10.1016/0891-5849(95)00005-i PMID: 7635356

54. Cabiscol E, Tamarit J, Ros J. Oxidative stress in bacteria and protein damage by reactive oxygen species. International Microbiology. 2010; 3(1):3–8.

55. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends in plant science. 2002; 7(9):405–10. https://doi.org/10.1016/s1360-1385(02)02312-9 PMID: 12294732

56. Angelova MB, Pashova SB, Spasova BK, Vassilev LS. Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. Mycological research. 2005; 109(2):150–8. https://doi.org/10.1017/s095375520401392 PMID: 15939099

57. Schirmer R, Schöhhammer T, Eisenbrand G, Krauth-Siegel R. Oxidative stress as a defense mechanism against parasitic infections. Free Radical Research. 1987; 3(1–5):3–12. https://doi.org/10.3109/10715768709069763 PMID: 3508442

58. Mathee K, Ciofu O, Sternberg C, Lindum PW, Campbell JIA, Jensen P, et al. Mucoid conversion of Pseudomonas aeruginosa by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microb. 1999; 145(6):1349–57.

59. Clifford DP, Repine JE. Hydrogen peroxide mediated killing of bacteria. Molecular and Cellular Biochemistry. 1982; 49(3):143–9. https://doi.org/10.1007/BF00231175 PMID: 6298593

60. Fukushima T, Tanaka K, Lim H, Moriyama M. Mechanism of cytotoxicity of paraquat. Environ Health Prev Med. 2002; 7(3):89–94. https://doi.org/10.1023/a:1012651065991 PMID: 21432289

61. Costerton JW, Stewart PS, Greenberg E. Bacterial biofilms: a common cause of persistent infections. Science. 1999; 284(5418):1318–22. https://doi.org/10.1126/science.284.5418.1318 PMID: 10334980

62. Leid JG. Bacterial biofilms resist key host defenses. Microbe. 2009; 4(2):66–70.