Irradiation effects of low temperature multi gas plasma jet on oral bacteria

Tahsin Raqib ABONTI1, Masato KAKU1, Shunichi KOJIMA1, Hiromi SUMI1, Shotoku KOJIMA1, Taeko YAMAMOTO1, Yuka YASHIMA1, Hidekazu MIYAHARA2, Akitoshi OKINO2, Toshitsugu KAWATA3, Kazuo TANNE1 and Kotaro TANIMOTO1

1 Department of Orthodontics, Applied Life Sciences, Hiroshima University Institute of Biomedical and Health Sciences, 1-2-3 Kasumi, Minami, Hiroshima 734-0037, Japan
2 Plasma Concept Tokyo, in Department of Energy Sciences, Tokyo Institute of Technology 4-14-13 Shinkoiwa, Katsushika, Tokyo 124-0024, Japan
3 Department of Orthodontics, Kanagawa Dental University, 82 Inaoka, Yokosuka, Kanagawa 238-8580, Japan

Corresponding author, Masato KAKU; E-mail: mkaku@hiroshima-u.ac.jp

The purpose of this study is to evaluate the sterilization effects of a newly developed low temperature multi gas plasma jet on oral pathogenic microorganisms (Streptococcus mutans [S. mutans], Lactobacillus fermentum [L. fermentum], Aggregatibacter actinomycetemcomitans [A. actinomycetemcomitans]). Plasma gas which generated from O2, N2, Ar and 50% (O2+N2) was irradiated to the microbes. Effect of O2 plasma irradiation on S. mutans under scanning electron microscopy (SEM) was also observed. O2 plasma was directly applied to dental plaque on human extracted tooth. Then, the depth of enamel resorption area was noted by nanoscale hybrid microscope. O2 had the best sterilizing effect for all microbes. The potent bactericidal effect of plasma irradiation was also observed by SEM. Decalcification of enamel was noted significantly lower in plasma irradiated tooth surface compared to no plasma exposure group. These findings revealed that multi gas plasma jet has great potential to be used for dental treatment.

Keywords: Multi gas plasma jet, Sterilization, Dental caries, Microorganisms

INTRODUCTION

As oral tissue and teeth are subjected to long term compromised oral environment due to plaque accumulation, caries, gingival inflammation1). Oral care is very crucial. However, sometimes, it is tremendous challenge for aged patients to clean their oral cavity. It is also difficult for patients with malocclusion to remove plaque owing to their crowding. So, to keep teeth and gingiva healthy, it is necessary to invent new oral care support device that can sterilize oral pathogenic microorganisms.

Recently, plasma sterilization has attracted increasing attention in dental community for low temperature atmospheric pressure plasma jets in order to sterilize the infected dental tissues2). Plasma is one of the four fundamental states of matter, others being solid, liquid and gas. Plasma is partially ionized gas in which a certain population of electrons are free rather than being bound to an atom or molecule. For plasma based sterilization, the gas used itself does not have any sterilizing effect. But when the gas changes to plasma, it obtains the properties that allow it to sterilize the substances that it touches3). It has been reported earlier about conventional low temperature plasma jet that it has limitations as plasma gas temperature is uncontrollable and plasma gas species are limited. For instances, thermal damage was observed on dentine surfaces due to increased local temperature which reached as high as 50°C, and was also pointed out that further irradiation of plasma on the dentine may lead to drying, cracks and damage to dentinal tubules, including odontoblasts and pulp tissue3). The purpose of this study is to evaluate the sterilizing effects of a newly developed low temperature multi-gas plasma jet using representative oral bacteria (Streptococcus mutans [S. mutans], Lactobacillus fermentum [L. fermentum], Aggregatibacter actinomycetemcomitans [A. actinomycetemcomitans]) to determine its possibility for clinical application.

MATERIALS AND METHODS

Newly developed low-temperature multi-gas plasma jet

A damage free, lightweight, portable multi gas plasma jet was developed by the Plasma Concept Tokyo, Department of Energy Science, Tokyo Institute of Technology, to conduct experiments in this study (Figs. 1A and B). It can generate stable atmospheric pressure plasma with oxygen, nitrogen, argon, carbon dioxide, helium, neon, air and mixtures of these gases. Gases used in our experiments are oxygen, nitrogen and argon. Gases become ionized at the exit of the source as a small jet with the flow rate of 5.00 L/min. Plasma gas temperature was maintained around 20°C.

Microorganisms and their culture conditions

We used 3 types of oral pathogenic microorganisms, S. mutans (NBRC13955 strain), L. fermentum (NBRC15885 strain) and A. actinomycetemcomitans (ATCC29522 strain). S. mutans and L. fermentum are gram-positive facultative anaerobic bacteria and are both highly acid producing species. S. mutans is one of the most implicated bacteria in smooth surface caries and considered to be major pathogen in dental caries3-6). L. fermentum has been associated with dental caries, and

Received Feb 12, 2016: Accepted Jul 4, 2016
doi:10.4012/dmj.2016-062  JOI JST.JSTAGE/dmj/2016-062
the quantity of Lactobacillus in saliva is used as a direct measure of caries risk, which is known as Lactobacillus count (Caries Test). A. actinomyctemcomitans is a gram-negative facultative anaerobic bacteria which is recognized as a major etiologic agent of aggressive periodontitis.

The microbes were stored frozen at −150°C. Prior to culture they were thawed and the microbes were cultured anaerobically in 3 mL of brain heart infusion broth, BHI (Becton, Dickinson and Company, MD, USA) in an anaerobic chamber overnight at 37°C. After this procedure, 100 µL of these cultures were inoculated into 10 mL of BHI cultured aerobically for 6 h at 37°C. Then another 10 µL was transferred to 10 mL of BHI and cultured overnight aerobically with shaking.

Experiment 1: Sterilization test on agar plates
The experiments in this study were conducted using the newly developed low temperature plasma jet using 3 types of gases which are O2, N2 and Ar and another mixture of gas 50% (O2+N2). After plating 100 µL of each microbial suspension onto the agar plate (EIKEN CHEMICAL, Tokyo, Japan), the surface was dried lightly. Tip of the tube of plasma jet was arranged at right angles to the surface of the agar plate and then plasma was irradiated from a distance of 2 and 20 mm respectively. Plasma exposure times were 10 and 60 s respectively and no plasma exposure was used as control. We tested 7 samples for each exposure time. After plasma irradiation, each oral pathogen was cultured on agar plates and then evaluation was determined by the number of living cells after plasma irradiation by colony forming unit (CFU) assay. We counted the number of colonies by use of a colony counter (Colony Counter 1.0, MICROTEC, Chiba, Japan) after taking photograph of agar plate.

Experiment 2: Observation of S. mutans morphology after O2 plasma irradiation by SEM
In order to observe morphology of S. mutans, bacteria were cultured following the previous method. After incubation, O2 plasma was irradiated for 60 s. Then the samples were harvested and washed by phosphate buffer saline (PBS, LSI Medience, Tokyo, Japan) and fix by 2% glutaraldehyde (KATAYAMA CHEMICAL, Osaka, Japan) for 2 h. After fixation, the samples were dehydrated in 50, 60, 70, 80, 90 and 100% ethanol for 10 min respectively. Then the sample was immersed in 2-methyl-2-propanol (NACALAI TESQUE, Kyoto, Japan) for 10 min. Afterwards, the samples were coated with Au-Pd and observed by scanning electron microscopy (SEM, JSM-7800F, JEOL, Tokyo, Japan).

Experiment 3: Plasma irradiation effect on human extracted tooth
In this experiment, human extracted teeth were used for direct application of plasma. The protocol was reviewed and approved by the Ethics Committee of Hiroshima University, and informed consent was obtained from each tooth donor. S. mutans were cultured following the previous culture method. Composite resin was used to make small partition wall (inside diameter: 2 mm) like structures on the enamel surface to isolate the area from activity of surrounding bacteria for both control group and plasma irradiated tooth surface (Fig. 2). Then bacteria were transferred to culture medium BHI broth with 10% sucrose along with the tooth immersed in it for 3 days for formation of plaque on tooth. After formation.

Fig. 1 The images of low temperature damage free multi-gas plasma jet (We received permission from Plasma Concept Tokyo for the publication of these photograph).

Fig. 2 Direct application of plasma on human extracted tooth. Composite resin was used to make small partition wall to isolate the area from activity of surrounding bacteria. S. mutans were cultured on the tooth in BHI broth with 10% sucrose. O2 plasma was directly applied after plaque formation.
of plaque, culture medium was changed every day to BHI broth with 10% sucrose for increasing the activity of microorganisms to enhance demineralization of enamel and finally formation of caries. For the control group only culture medium was changed every day and no plasma exposure was used. O2 plasma irradiation for 60 s per day was continued for 21 consecutive days. The depth of enamel resorption area was noted by a nanoscale hybrid microscope (VN-800, Keyence, Osaka, Japan).

**Statistical analysis**
Statistical analysis for sterilization effect was evaluated using analysis of variance and multiple comparison tests (sheffe). Differences between the mean values of the depth of enamel resorption from control and plasma irradiation group were examined by student’s t-test. A confident level of \( p<0.05 \) is defined as statistical significant.

**RESULTS**

**Experiment 1: Sterilization effect on agar plates**
1. Sterilization effect on *S. mutans* (Fig. 3)
By the direct application of plasma jet on agar plates the sterilization effect was observed. As shown in Fig. 3, a time dependent increase of the sterilizing effect was observed for *S. mutans* when the values were compared between 10 and 60 s. Statistical difference was observed in 10 s, 2 mm, 10 s, 20 mm, 60 s, 2 mm and 60 s, 20 mm groups compared to control after application of O2 plasma \( (p<0.01) \). Sterilization effect of N2 plasma is shown in Fig. 3. Compared to the control of no irradiation sample 10 s, 2 mm sample and 10 s, 20 mm sample were not significantly different. Whereas, 60 s, 2 mm and 60 s, 20 mm sample showed significant reduction of CFU \( (p<0.01) \). Irradiation effect of Ar plasma is shown in Fig. 3. There was no significant reduction of CFU and no significant statistical difference between the control and the other tested samples. There was significant reduction of the survival of *S. mutans* after irradiation of 50% \( (\text{O}_2+\text{N}_2) \) plasma in the tested sample of 60 s, 2 mm and 60 s, 20 mm \( (p<0.01) \). The rest of the samples showed no significant difference.

2. Sterilization effect on *L. fermentum* (Fig. 4)
A time dependent reduction of growth was observed after irradiation of \( \text{O}_2 \), \( \text{N}_2 \) and 50% \( (\text{O}_2+\text{N}_2) \) plasma. Significantly reduction was shown like *S. mutans* after irradiation of \( \text{O}_2 \) plasma \( (p<0.01) \) and complete inhibition of growth was observed in the tested sample of 60 s, 20 mm. Among the tested samples, \( \text{N}_2 \) plasma showed best result in 60 s, 20 mm group. It clearly showed time dependent reduction of growth. After irradiation of Ar plasma, reduction of survival of *L. fermentum* but no
significant statistical difference in all tested samples were observed as compared to the control. After irradiation of 50% (O₂+N₂) plasma, there was no significant difference among all the tested samples except 60 s, 20 mm sample which showed inhibition of growth of L. fermentum (p<0.01).

3. Sterilization effect on A. actinomycetemcomitans (Fig. 5)
There was zero survival of the microorganisms after irradiation of O₂ plasma in 60 s, 20 mm sample. Statistical difference could be observed in 60 s, 20 mm sample after irradiation of N₂ plasma (p<0.01). After

Fig. 6 Observation of S. mutans morphology after O₂ plasma irradiation by SEM (A: control group, B: plasma irradiation group).

Fig. 7 Direct application of plasma on human extracted tooth (A and B: control group, C and D: plasma irradiation group).
irradiation of Ar plasma, there was little reduction of survival of \textit{A. actinomycetemcomitans}, but there was no significant statistical difference compared to control. There was statistical reduction of growth in both 60 s, 2 mm and 60 s, 20 mm sample after irradiation of 50% (O\textsubscript{2}+N\textsubscript{2}) plasma ($p<0.01$).

**Experiment 2: Observation of \textit{S. mutans} morphology after O\textsubscript{2} plasma irradiation by SEM**

As shown in Fig. 6, plasma irradiation on \textit{S. mutans} partially resulted in a significant alteration in cell size and morphological changes when compared with the untreated controls. In the control group with no plasma exposure (Fig. 6A), the cell structure of the bacteria appears to be intact with no damage. Whereas, damage on the cell walls with biofluid leakage and fragments of cells were noticed in the plasma irradiated group (Fig. 6B).

**Experiment 3: Effect of plasma Irradiation on human extracted tooth**

The effect of the enamel resorption area in this experiment was observed by the hybrid nanoscale microscope. As it is shown in Figs. 7A and B, the control group with no plasma irradiation shows deep cavities on the enamel surface representing decalcification and demineralization of enamel. The depth of enamel resorption area was counted 127 µm for the control group (Fig. 8). On the other hand, the plasma irradiated enamel surface appears to be more flat compared to control (Figs. 7C and D). Significantly lower enamel resorption area was shown, \textit{i.e.} 27 µm compared to control ($p<0.01$, Fig. 8).

**DISCUSSION**

Dental treatment changes the oral environmental factors. It is necessary to maintain the balance between the caries risk factors and periodontal disease prevention during treatment. So there is need to develop intraorally applicable sterilization techniques and newly developed low temperature multi gas plasma jet represents such technique. In this study, we evaluated the efficacy of plasma on disinfection of microorganisms and considered the possibility of applying the technique for clinical use.

**Sterilization test on agar plates**

There have been reports on the effectiveness of other plasmas for sterilization of microorganisms by using tests of inhibitory zone assays on agar plates\textsuperscript{10}. In this study, we used different types of gas and mixture of gases to find out the efficiency of multi gas plasma jet in sterilizing microorganisms in dry phase. The spot diameter of the plasma at the point of contact with the agar plate was set to 2 and 20 mm respectively. Plasma sterilization from the distance of 20 mm was significant irrespective of all the microorganisms and all the gases used. Possible reason for that might be that the plasma could spread onto more surface area on the agar plates from 20 mm distance compared to 2 mm distance. Another reason might be that when the plasma jet was set higher it could come in more contact with surrounding air and possibly could produce more reactive species that had more sterilizing effect on microorganisms. After irradiating the samples with O\textsubscript{2} and N\textsubscript{2} plasma for 60 s, the CFU was counted very low or almost zero in some samples for each type of microorganisms. This demonstrates that the multi gas plasma jet when directly applied is capable of rapidly sterilizing microorganisms present on the agar plate.

O\textsubscript{2} plasma had the best sterilizing effect on all three types of bacteria. Plasma gas containing higher oxygen concentration have been associated with increased levels of microbial inhibition due to higher level of oxygen based active species such as atomic oxygen and ozone\textsuperscript{11}. The active species formed during plasma discharge, hydroxyl (OH), singlet oxygen, ozone and radicals can initiate lipid peroxidation thus producing shorter chain fatty acyl compounds. These products include alkanes, ketones, epoxides and aldehydes\textsuperscript{12-14}. The short chains of charged fatty acids have a lower ability to rotate within the membrane and increase the fluidity of the membrane\textsuperscript{12,15} which can result in distraction of structural membrane integrity\textsuperscript{16}.

Atmospheric pressure Non Thermal Plasma (NTP) has been observed to have a killing effect on many kinds of microorganisms and is therefore becoming the technology of choice for decontamination purposes. Studies have outlined three basic mechanisms attributed to cell death by plasma. These are cell surface etching induced by reactive species formed during plasma generation, volatilization of compounds and intrinsic photodesorption of ultraviolet (UV) photons and the destruction of genetic material. These deactivation
mechanisms cause several reactions in bacterial cells including lipid peroxidation of poly-unsaturated fatty acids, oxidation of amino acids and DNA oxidation. Also in the present study of SEM observation, damage on the cell walls with biofluid leakage and fragments of cells of S. mutans after plasma irradiation were detected. These results indicate that the mechanism on the antibacterial effect of plasma irradiation is due to destruction of cell walls by cell surface etching.

Although a sterilizing effect can be caused by UV radiation produced which acts by disturbing the genetic material of microorganisms, several studies have shown that the UV radiation produced by NTP’s does not play a major part in inactivation of microorganisms. Instead it is the effect of reactive species produced by NTP’s that are believed to be the major cause of microbial cell death. The reactive species in non-thermal atmospheric plasmas have been found to be nitrogen and oxygen based species such as atomic oxygen, ozone, nitrogen oxide and hydroxyl. These active species play a crucial role in the microbial inactivation mechanism, since they directly interact with the bacterial membrane. These active species have short life in the gas phase thereby disappearing in milliseconds after they are produced and can also dissolve in liquids.

In this study it was noted that A. actinomycetemcomitans was weak compared to other two microorganisms. A. actinomycetemcomitans was rapidly sterilized within a short period of time as 10 s irrespective of all the gases used and without showing dependency towards distance. It is known that vegetative cells tend to be more susceptible to plasma application than spores, as is generally true of microbial cell death. The reactive species in non-thermal atmospheric plasmas have been found to be nitrogen and oxygen based species such as atomic oxygen, ozone, nitrogen oxide and hydroxyl. These active species play a crucial role in the microbial inactivation mechanism, since they directly interact with the bacterial membrane. These active species have short life in the gas phase thereby disappearing in milliseconds after they are produced and can also dissolve in liquids.

In this study it was noted that A. actinomycetemcomitans was weak compared to other two microorganisms. A. actinomycetemcomitans was rapidly sterilized within a short period of time as 10 s irrespective of all the gases used and without showing dependency towards distance. It is known that vegetative cells tend to be more susceptible to plasma application than spores, as is generally true of microbial cell death. The reactive species in non-thermal atmospheric plasmas have been found to be nitrogen and oxygen based species such as atomic oxygen, ozone, nitrogen oxide and hydroxyl. These active species play a crucial role in the microbial inactivation mechanism, since they directly interact with the bacterial membrane. These active species have short life in the gas phase thereby disappearing in milliseconds after they are produced and can also dissolve in liquids.

Observation of S. mutans morphology after O₂ plasma irradiation by SEM

In the control group, the cell structure of S. mutans showed no damage. However, morphological changes such as biofluid leakage and fragments of cells were observed after O₂ plasma irradiation. These results strongly suggest that the sterilization effect for microorganisms which were observed on agar plates in this study was due to destruction of cell membrane by plasma irradiation.

Plasma irradiation effect on human extracted tooth

Deep carries on the enamel surfaces were observed in the control group without plasma irradiation showing decalcification and demineralization, although enamel surface with plasma irradiation showed significantly lower enamel resorption area. These results strongly suggest that O₂ plasma has great potential for dental treatment.

CONCLUSIONS

The major findings of the present studies of the sterilization effect of newly developed low temperature multi gas jet on oral pathogenic microorganisms are as follows:

1. Present study revealed that multi gas plasma jet was effective in sterilizing all three oral pathogenic microorganisms that we examined on agar plates.
2. Time dependent and distance dependent tendency in the sterilizing effect of plasma was significant.
3. The most effective plasma gas was O₂ among all the other gases used.
4. SEM images indicated rapid and effective deactivation of S. mutans with the application of plasma O₂.
5. Enamel resorption area was noted significantly lower in plasma irradiated tooth surface compared to control.

The favorable results of the present study indicate that the newly developed plasma jet has a great potential for being used in the field of dental treatment.

ACKNOWLEDGMENTS

We thank Dr. SUGAI and Dr. KAYAMA, Laboratory of Bacteriology, Graduate School of Biomedical Sciences, Hiroshima University for their support in conducting our research. This work was supported by Promotional project for Research of Detection system of Biological Reaction and Life-maintenance Mechanism at Hiroshima University.

REFERENCES

1) Bollen AM, Cunha-Cruz J, Bakko DW, Huang GJ, Hujoel PP. The effects of orthodontic therapy on periodontal health, a systematic review of controlled evidence. J Am Dent Assoc 2008; 139: 413-422.
2) Yamazaki H, Ohshima T, Tsubota Y, Yamaguchi H, Jayawardena JA, Nishimura Y. Microbicidal activities of low frequency atmospheric pressure plasma jets on oral pathogens. Dent Mater J 2011; 30: 384-391.
3) Collins CH, Lyne PM. In: Collins CH, Lyne PM, Grange JM, Falkinham IIIJO editors. Collins and Lyne’s microbiological methods. New York: Oxford University Press Inc; 1984.
4) Loesche WJ. In: Charles C editor. A treatable infection in Dental caries. Illinois: Thomas Publisher; 1982.
5) Keene HJ. Sampling of cariogenic microorganisms in human populations. Oral Microbiol Immunol 1986; 1: 7-12.
6) Schaeken MJ, Van der Hoeven JS, Franken HC. Comparative recovery of Streptococcus mutans on five isolation media, including a new simple selective medium. J Dent Res 1986; 65: 906-908.
7) Shen S, Samaranayake LP, Yip HK. In vitro growth, acidogenicity and cariogenicity of predominant human root caries flora. J Dent 2004; 32: 687-698.
8) Sumney DL, Jordan HV, Englander HR. The prevalence of root surface caries in selected populations. J Periodontol
9) Slots J, Ting M. Actinobacillus actinomyctecomitans and Porphyromonas gingivalis in human periodontal disease: occurrence and treatment. J Periodontol 1999; 20: 82-121.

10) Chunqi J, Meng-Tse C, Schaudinn C, Gorur A, Vernier PT, Costerton JW. Pulsed atmospheric-pressure cold plasma for endodontic disinfection. IEEE Trans Plasma Sci 2009; 37:1190-1195.

11) Kuzmichev AI, Soloshenko IA, Tsiolko VV, Kryzhanovsky VI, Bazhenov VY, Mikhno IL, Khomich VA. Feature of sterilization by different type of atmospheric pressure discharges. Proceedings on International Symposium on High Pressure Low Temperature Plasma Chemistry; 2009. p. 402-406.

12) Mead J. In: Pryor WA, editor. Free radical mechanisms of lipid damage and consequences for cellular membranes. Free radicals in biology. New York: NY Academic Press; 1976. p. 51-68.

13) Benedetti A, Comporti M, Fulceri R, Esterbauer H. Cytotoxic aldehydes originating from the peroxidation of liver microsomal lipids. Biochim Biophys Acta 1984; 792: 172-181.

14) Kappus H. In: Sies H, editor. Oxidative stress. New York: Academic Press; 1985. p. 273-310.

15) McElhaney RN. In: Benga G, editor. Structure and properties of cell membranes. Boca Raton: CRC Press; 1985. p. 75-91.

16) Korachi M, Aslan N. Low temperature atmospheric plasma for microbial decontamination, Microbial pathogens and strategies for combating them. J Sci Educ Technol 2013; 1: 453-459.

17) Moisan M, Barbeau J, Crevier MC, Pelletier J, Philip N, Saoudi B. Plasma sterilization, methods and mechanisms. Pure Appl Chem 2002; 74: 349-358.

18) Korachi M, Gürol C, Aslan N. Atmospheric plasma discharge sterilization effects on whole cell fatty acid profiles of Escherichia coli and Staphylococcus aureus. J Electrostat 2010; 68: 508-512.

19) Korachi M, Aslan N. The effect of atmospheric pressure plasma corona discharge on pH, lipid content and DNA of bacterial cells. Plasma Sci Technol 2011; 13: 99-105.

20) Herrmann HW, Henins I, Park-J, Selwyn GS. Decontamination of chemical and biological warfare (CBW) agents using an atmospheric pressure plasma jet (APPJ). Phys Plasmas 1999; 1: 2284-2289.

21) Yamamoto M, Nishioka M, Sadakata M. Sterilization using a corona discharge with HO droplets and examination of effective species. Proceedings of 15th International Symposium on Plasma Chemistry 2001. p. 743-751.

22) Korachi M, Turan Z, Senturk K, Sahin F, Aslan N. An investigation into the biocidal effect of high voltage AC/DC atmospheric corona discharges on bacteria, yeasts, fungi and algae. J Electrostat 2009; 67: 678-685.

23) Gadri BR, Roth JR, Montie TC, Kelly-Wintenberg K, Tsai PPy, Helfritch DJ, Feldman P, Sherman DM, Karakaya F, Chen ZY. Sterilization and plasma processing of room temperature surfaces with a one atmosphere uniform glow discharge plasma. Surf Coat Technol 2000; 131: 528-542.