Non-Thermal Atmospheric Plasma for Endodontic Treatment

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Abstract: Gas discharge plasma is being explored nowadays for its application as an alternative to the conventional sterilization and disinfection techniques in medical sciences. We have developed the non-thermal atmospheric plasma torch to study the effect of plasma treatment on the growth rate of E. faecalis culture and biofilms. E. faecalis treated with plasma was then compared with helium gas exposed and chlorohexidine treated cultures and biofilms. All the results are analysed for significance ($P < 0.001$) using ANOVA and TUCKEY'S test. Optical emission spectroscopy technique has been employed in. situ to identify the species interacting with the samples. It is found that atmospheric non-thermal plasma proves to be a promising alternative to traditional disinfectants for disinfection during endodontic treatment.

1 INTRODUCTION

Recently, cold plasma is being explored for its application as an alternative to the conventional sterilization and disinfection techniques in medical field. Amongst the different types of plasmas, studies on atmospheric non-thermal plasma has gained importance in the medical field due to its property of being functional at room temperature and atmospheric pressure; unlike low pressure cold plasmas. The antimicrobial property of atmospheric plasma has been demonstrated against several bacteria for example *Escherichia coli*, *Candida albicans*, *Streptococcus mutans*, *Bacillus subtilis* etc (Hong, 2009).

In the field of dentistry, microorganisms and their by-products are considered to be the major cause of pulp and periradicular pathosis. The objective of endodontic therapy is to i] remove diseased tissue, ii] eliminate bacteria present in the canals and dentinal tubules and iii] prevent post endodontic recontamination. Hence, a major objective in root canal treatment is to disinfect the entire root canal system and to eliminate all the possible sources of infection. This can be accomplished by using mechanical instrumentation and chemical agents, in conjunction with medication of the root canal system between treatment sessions. Inspite of these treatments, the survival of microorganisms in the apical portion of root filled teeth is still the major cause of endodontic failure. Studies have revealed that this increased resistance is due to the formation of biofilms by the microorganisms. Microorganisms like *Enterococcus faecalis*, *Streptococcus mutans*, *Candida albicans* can adhere to the root canal walls and form communities organized in biofilm which makes them more resistant to phagocytosis, antibodies, and antimicrobials. This is due to the presence of exopolysaccharides in comparison with non-biofilm producing organisms (Sabeena, 2010). Also, due to the presence of dentin tubules; the disinfection of dentin posses special challenges during caries therapy. Conventionally disinfection is achieved by i] invasive removal, ii] use of chemicals like chlorhexidine. But these treatments do not disinfect the dental tubules completely. Contact dermatitis is a common adverse reaction to chlorhexidine. Chlorhexidine is also liable to cause desquamative gingivitis, discoloration of tongue and teeth or dysgeusia (distorted taste). Plasma being in the gaseous form would have a better reach in the confined and tortuous root canal system & may prove to be a better adjunct to root canal instrumentation for canal sterilization. Hence,

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inactivation of microorganisms using plasma has attracted much attention recently.

Numerous in vitro studies have been conducted on the effects of atmospheric plasma on pathogens like *Streptococcus mutans*, *Candida albicnas*, *Chromobacterium violaceum* etc. Amongst the various oral pathogens *Enterococcus faecalis* is one of the primary organisms in patients with post treatment endodontic infection (Mohammadi, 2009). *E. faecalis* is known to form intracanal biofilms, periapical biofilms and biomaterial centered infection. Inspite of being one of the important infection causing organism, relatively few reports are available that describe the efficacy of atmospheric plasma against *E. faecalis*. Hence, this study was aimed at evaluating the inhibitory effect of atmospheric plasma against *E. faecalis* culture and biofilms.

The paper reports the use of He (atmospheric pressure) as a plasma generating gas which enables the formation of active reactive species useful for bactericidal properties.

2 MATERIALS AND METHODS

Atmospheric non-thermal plasma torch is found to be useful for varieties of applications. Its use for dental applications is an emerging field of research. In the present article we report on generation of miniaturize plasma torch using helium as plasma forming gas. Fig 1 shows the schematic of the atmospheric pressure non thermal plasma torch which consisted of a tungsten cathode in the form of thin wire of axially fitted into a cylindrical glass tube with a fine nozzle. The anode was a stainless steel plate which formed the base below the microtitre plate. Helium was made to flow at a flow rate of 1 lpm through the torch system so as to reach the bacterial culture placed inside the microtitre plate. Pulsed dc voltage of 24 kV was used to operate the torch. The plasma plume 1-2 mm in diameter extended outside the nozzle upto a distance of 2-3 cm and reached the *E. faecalis* culture. Helium having a low ionization potential serves as a suitable ionizing medium and helps in extracting the plasma plume to larger distances. Compared to other gas plasmas it is easy to obtain plume of few centimeter by using helium gas as plasma forming gas (Sladek, 2004). Figure 2 shows the photograph of the plasma torch in operation, treating culture and biofilms in microtitre plate

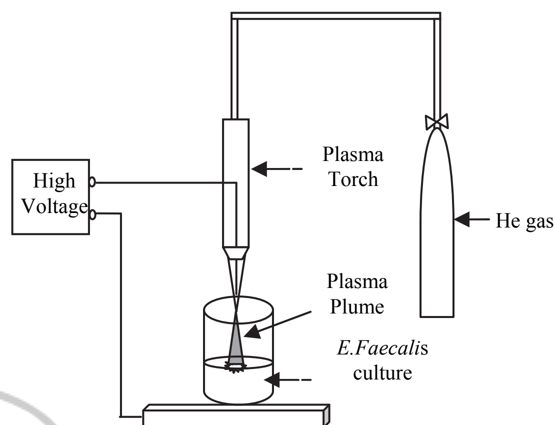

Figure 1: Shows the schematic of the atmospheric pressure non thermal plasma torch.

The glow in the plasma is due to electron excitation de-excitation and ionization of gas atoms, so that it serves as a visual indicator of the presence of energetic electrons and photons. These energetic electrons generate radicals by dissociating gas molecules such as H_2O and O_2, or by generating metastable He ions that probably dissociate H_2O molecules. The air mixed with water molecules help in generating the reactive OH and O radicals which helps in killing the *E. faecalis* bacteria (J. Goree, 2006).

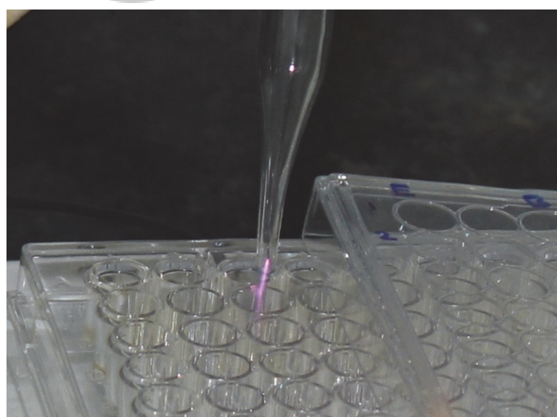

Figure 2: Photograph of actual atmospheric non-thermal plasma torch while treating the samples.

Enterococcus faecalis (NCIM 5025) was procured from National Collection of Industrial Microorganisms, Pune. The strain was maintained on MRS (de Man, Rogosa and Sharpe) medium (composition per liter: peptone 10 g, meat extract 10 g, yeast extract 5 g, sodium acetate 5 g, dipotassium phosphate 2 g, ammonium citrate 2 g, magnesium sulfate 0.2 g, manganese sulfate 0.05 g, glucose 20

g, tween 80 1 g) slants and stored at 4°C.The effect of atmospheric plasma against *E. faecalis* culture was monitored as follows. 100 µl of culture was placed in each well of 96 well microtitre plate. The culture was then treated with atmospheric plasma for 2 minutes. The wells were grouped into 4 groups viz. group A-helium treated, group B, plasma treated and group C- chlorohexidine treated D- control group with group size of 30 wells per group. *E. faecalis* culture was treated with chlorohexidine for 2 minutes by adding 100 µl 0.2 % chlorohexidine solution to the wells and subsequent removal of the solution after 2 minutes. The viability of the cells was checked by Triphenyl tetrazolium chloride (TTC) assay.

Preparation of bio-films: E. faecalis was inoculated in MRS broth and incubated at 37°C at 120 rpm for 24 hours. 100 µl of culture (OD 1.0 according to McFarland's scale, colony forming unit (CFU) 4× 108 / ml) was added in each well of 96-well flat base microtitre plate. The plate was incubated at 37°C for 1 hour in order to allow the organisms to adhere to the surface of the wells. After 1 hour, remaining culture form the wells was replaced by 100 µl of fresh MRS broth and the plate was further incubated at 37°C for 24 hours. After incubation, the excess medium form the wells was removed and the biofilms then treated with atmospheric plasma for 2 minutes. The experiment was divided into 4 groups as mentioned above.

2.1 Viability Assay

The viability of the organisms was determined using the TTC viability assay described by *C E* Nwanyanwu with some modifications (Nwanyanwu et al, 2011). 100 µl of 1 % (w/v) solution of TTC prepared in sterile distilled water was added in the wells along with 100 µl of MRS broth following the treatments. The plates were incubated at 37°C overnight. The color change was measured at 490 nm using Elisa plate reader (Bio Rad model no: IMark)

All the results are analysed for significance ($P < 0.001$) using ANOVA and TUCKEY'S test. Optical emission spectrometer (OES) model HR-4000 (manufactured by Ocean Optics) was employed for identification of the active ionic species present in the plasma. This spectrometer is having detector (model TCD1304AP) with linear CCD array and the range of detection 200-1100 nm. Other specifications of the spectrometer are availability of shutter mode, fiber optical input and the optical resolution is ~0.03nm (FWHM).

3 RESULTS AND DISCUSSION

E. faecalis culture was exposed to atmospheric non-thermal helium plasma for 2 min. Similarly, culture was also treated with chlorohexidine for 2 minutes. The results of the viability assay were then compared for the two processes as shown in figure 3. Figure shows the mean bacterial count in terms of optical density in all the four groups. The wells in the control group and the wells treated with helium exhibited no change in the optical density (OD 1.0) indicating 100 % survival of the culture. However significant reduction in optical density was observed in the wells exposed to chlorohexidine (OD 0.7) and plasma (OD 0.6). Inhibitory effects of atmospheric plasma have also been reported on *E. faecalis* culture by Cao et al (2011). There the authors had exposed the culture to helium -oxygen plasma for 5, 10 and 15 min. The post exposure viability was determined by measuring the zone of inhibition. Effects of atmospheric plasma, helium and chlorohexidine on *E. fecalis* biofilms adhered on wells of the microtitre plates were determined by TTC viability assay. The results were similar to those observed for the culture. Chlorohexidine and plasma treated biofilms showed decrease in optical density.

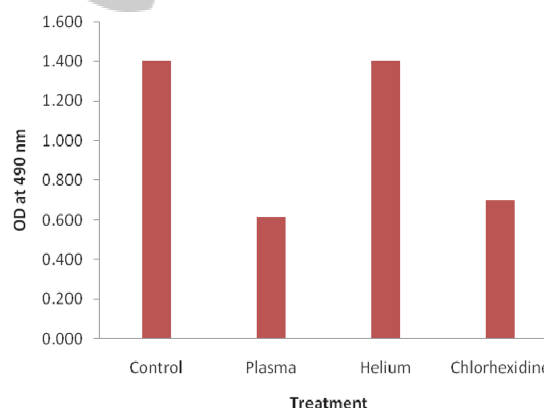

Figure 3: Viability Assay of effect of plasma treatment and chlorohexidine treatment on *E. faecalis* culture.

It was 0.5 for chlorohexidne, 0.47 for plasma as compared to 0.8 of control and helium treated biofilms. This clearly indicated reduction in viability of bacteria in biofilms (fig3).

In order to confirm the presence of the reactive species we have carried out the spectral identification using optical emission spectroscopy. Fig 4 shows the OES spectrum recorded during the plasma exposure of the *E. faecalis* culture. Optical

emission spectrum shows that the species present in the plasma include helium, atomic oxygen (O), Ozone (O_3), and OH radicals.

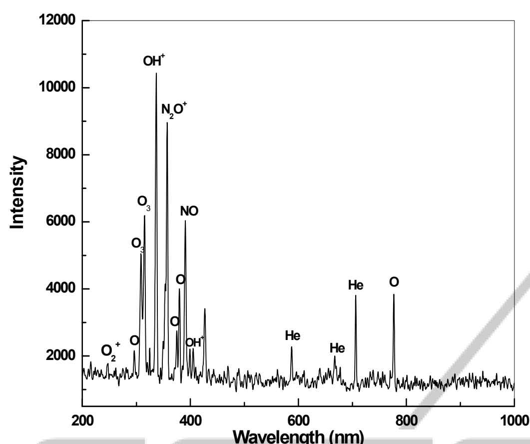

Figure 4: Optical emission spectrum of helium plasma generated by atmospheric non-thermal plasma torch.

Fig 5 shows helium gas did not show inhibitory effect on bio films. Inhibitory effects of atmospheric plasma have also been examined on *Chromobacterium violaceum* (Jonathan, 2009) and on *Streptococcus mutans* biofilms (Sladek, 2007). Du et. al. (2011) have also reported reduction in viability of *E. faecalis* biofilms after treatment with atmospheric plasma. The authors determined viability using confocal laser scanning microscopy. Cao et al (2011) have reported antibacterial effects of atmospheric plasma on *E. faecalis* biofilms prepared on nitrocellulose membrane using SEM. Our results of 2 min exposure leading to significant reduction in the viability suggest that this technique can be used as an adjunct for endodontic therapy in dentistry. Further research in this line could prove its potential as an alternative for traditional procedures of disinfection.

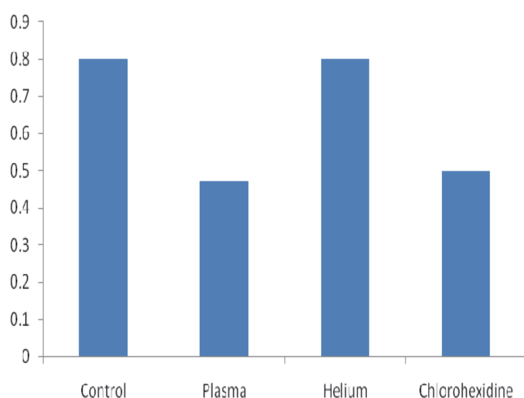

Figure 5: Shows inhibitory effect on *E. faecalis* biofilms.

Oxygen radical and OH radicals are known to be bactericidal. It reacts with the cell wall of the bacteria due to which it gets ruptured and cytoplasm comes outside cause the death of the bacteria.

4 CONCLUSIONS

Atmospheric plasma proves to be a promising alternative to traditional disinfectants for disinfection during endodontic treatment. Using plasma will be beneficial mainly due to its gaseous nature, improved dispersion of disinfectant in the dentinal tubules, better reach in the tortuous canals and no dysgeusia (distorted taste). Hence, application of atmospheric non-thermal plasma can be a time saving method since post disinfection clean up procedures can be minimized during endodontic treatments.

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