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Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro – proof of principle experiment

Ina Koban[1], Birte Holtfreter[1], Nils-Olaf Hübner[2], Rutger Matthes[2], Rabea Sietmann[3], Eckhard Kindel[4], Klaus-Dieter Weltmann[4], Alexander Welk[1], Axel Kramer[2] and Thomas Kocher[1]

[1]Department of Restorative Dentistry, Periodontology and Endodontology, Unit of Periodontology, Ernst-Moritz-Arndt University Greifswald, Germany; [2]Institute for Hygiene and Environmental Medicine, Ernst-Moritz-Arndt University Greifswald, Germany; [3]Institute of Microbiology, Ernst-Moritz-Arndt University Greifswald, Germany; [4]Leibniz Institute for Plasma Science and Technology, Greifswald, Germany

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Abstract

Aim: Dental biofilms play a major role in the pathogenesis of peri-implant mucositis. Biofilm reduction is a pre-requisite for a successful therapy of peri-implant mucosal lesions. In this study, we evaluated the effect of three different plasma devices on the reduction of *Streptococcus mutans* (*S. mutans*) and multispecies human saliva biofilms.

Material and Methods: We assessed the efficacy of three different non-thermal atmospheric pressure plasma devices against biofilms of *S. mutans* and saliva multispecies grown on titanium discs in vitro in comparison with a chlorhexidine digluconate (CHX) rinse. Efficacy of plasma treatment was determined by the number of colony forming units (CFU) and by scanning electron microscopy. The results were reported as reduction of CFU ($CFU_{untreated} - CFU_{treated}$).

Results: The application of plasma was much more effective than CHX against biofilms. The maximum reduction of CHX was 3.36 for *S. mutans* biofilm and 1.50 for saliva biofilm, whereas the colony forming units (CFU) reduction of the volume dielectric barrier discharge argon plasma was 5.38 for *S. mutans* biofilm and 5.67 for saliva biofilm.

Conclusions: Treatment of single- and multispecies dental biofilms on titanium discs with non-thermal atmospheric pressure plasma was more efficient than CHX application in vitro. Thus, the development of plasma devices for the treatment of peri-implant mucositis may be fruitful.

Key words: chlorhexidine; human saliva biofilm; non-thermal plasma; *Streptococcus mutans*

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Conflicts of interest and source of funding statement

The authors declare that they have no conflict of interests.

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All titanium discs were kindly provided by Straumann.

Pockets at dental implants communicate with the oral cavity. In contrast to teeth, collagen fibres do not insert on the implant surface; only a soft tissue collar ensures a bacterial barrier against infection of the underlying tissue from oral cavity. Therefore, the peri-implant mucosa with its subepithelial connective tissue and the epithelium, serving as a peri-implant soft tissue seal, is considered as a locus minoris resistentiae. Several studies have shown that insufficient oral hygiene and insufficient maintenance can lead to perimucositits, which is considered as a precursor of periimplantitis for which no successful treatment solutions have been found yet (Berglundh et al. 2002). Hence, early treatment of peri-implant mucositis is essential for a healthy long-term survival of the implants. There seems to be no difference between the aetiology of plaque-induced gingivitis and peri-implant mucositis. Both diseases are caused by a multispecies biofilm. Whereas the single species of a mixture

of bacteria could not induce experimental abscesses, the combination of these species could do it (Mombelli 1999).

Plasma represents the fourth state of matter after solid, liquid, and gaseous. It is formed when a gas is ionized (Morfill et al. 2009). It is electrically neutral, composed of ions, electrons, vacuum ultraviolet and ultraviolet irradiation, free radicals, and chemically reactive neutral particles. The short lifespan of these species is desirable as they do not remain after the treatment is completed (Goree et al. 2006). Tissue-compatible temperatures of plasma may allow its application in the mouth. Inactivation or bio-decontamination of bacteria and sterilization with plasma have received much attention in recent years (Foest et al. 2006). Plasma is known to inactivate planktonic bacteria, yeast, and spores (Fridman et al. 2007, Kolb et al. 2008, von Woedtke et al. 2008, Rupf et al. 2010) and it has a dose-dependent antimicrobial effectivity (Sladek & Stoffels 2005). New medical devices using atmospheric pressure plasmas are under development. Plasma application may be a new technology to remove biofilms and it appears promising to investigate its effects on biofilms on dental implants.

Often efficacy of antimicrobial therapies is examined on planktonically grown bacteria, because these are convenient to work with instead of a complex biofilm, where antimicrobial resistance is probably several magnitudes higher (Sedlacek & Walker 2007). To mimic clinical reality, antimicrobial efficacy should be tested in a biofilm model, but only a few investigations have been published so far with most of them limited to monospecies biofilms (Wilson 1996, Eick et al. 2004, Hoiby et al. 2010).

In the present study, the antimicrobial potential of three different plasma devices [plasma jet, hollow electrode dielectric barrier discharge plasma, and volume dielectric barrier discharge (VDBD) plasma] was tested on biofilms of *Streptococcus mutans* in vitro and multispecies human saliva biofilms ex vivo.

Material and Methods

Bacterial strains/biofilm formation

In this study, *S. mutans* (DSM 20523, German collection of microorganisms and tissue culture cells, Braunschweig, Germany) was used. We used unstimulated saliva of healthy donors ($n = 6$, pooled saliva, age 20–30 years, nonsmokers) as a source of oral microbiota. Saliva donors did not take any medication 3 months prior the study and did not have active carious lesions or periodontal disease. The study was approved by the local ethics committee.

Biofilms were cultured on machined titanium discs with a diameter of 5 and 1 mm thickness (Institut Straumann AG, Basel, Switzerland). Biofilm cultivation was done as described before (Koban et al. 2010), *S. mutans* was grown overnight at 37°C on Columbia sheep blood agar (BBL™, BD, Heidelberg, Germany). One inoculation loop of this culture was re-suspended into 30 ml brain heart infusion (BHI) (BBL™, BD, Heidelberg, Germany) complemented with 1% sucrose (Merritt et al. 2003). The sterile titanium discs were positioned in 96-well microtitre plates (Techno Plastic Products AG, Trasadingen, Switzerland), covered with 100 μl microorganism suspension or saliva, and incubated aerobically at 37°C. For *S. mutans*, we deliberately used no surface coating with salivary proteins, because this reduces the contact angle of titanium and, consequently, there would be a reduced adhesion of *S. mutans* (Fujiokahirai et al. 1987). Every 24 h BHI was changed. After 48 h the medium was drawn off, the discs were washed with 0.9% NaCl solution and transferred into a new, sterile microtitre plate.

After plasma treatment, titanium discs were placed into wells with 200 μl 0.9% NaCl solution and the biofilm was removed by treatment in an ultrasonic bath (Branson 2510, 130 W, 42 kHz, Dietzenbach, Germany). Serial dilutions of the re-suspended biofilm solution were made by transferring 0.1 ml of the resultant suspension to 0.9 ml of fresh 0.9% NaCl solution. Afterwards an aliquot portion of 0.1 ml from each dilution was plated on BHI agar plates (BBL™, BD) and incubated at 37°C for 48 h. The colonies were counted and expressed as CFU/ml.

The $\log_{10}$ reduction factor (RF) for each treatment method was calculated according to the formula (Müller et al. 2003): $RF = \log_{10} n_c - \log_{10} n_u$; where n_c = number of viable cells (CFU) in the re-suspended biofilm solution of untreated control (in the presence of 0.9% NaCl solution); n_u = number of viable cells (CFU) in the re-suspended biofilm solution after contact with chlorhexidine or plasma.

Antiseptic treatment

Chlorhexidine digluconate (CHX) was used as an aqueous solution 0.1% (Fagron GmbH & Co KG, Barsbüttel, Germany). This is a standard concentration used in dental clinics and commercial mouth rinse solutions. The discs were covered with 100 μl of the antiseptic and incubated for 1, 2, 5, or 10 min. After the incubation period CHX was drawn off, and the antiseptic effect was stopped by adding 100 μl inactivator (Lipofundin MCT 20%, B. Braun, Melsungen, Germany). This inactivation was verified by the quantitative suspension test according to DIN EN 1040 (DIN EN 1040 2005) (data not shown).

Plasma treatment

For plasma generation we used three different devices, developed by the Leibniz Institute for Plasma Science and Technology (INP, Greifswald, Germany): an atmospheric pressure plasma jet (kINPen 09), a hollow dielectric barrier discharge electrode (HDBD), and a volume dielectric barrier discharge (VDBD) [Fig. 1 (Koban et al. 2010)].

Plasma jet (kINPen 09)

The plasma jet consists of a handpiece for generation of a plasma at atmospheric pressure, a DC power supply and a gas supply unit [Fig. 1a,b (Foest et al. 2005)]. For our experiments the argon (Ar) gas flow was set to 5 slm (standard litres per minute). The flow rate was controlled by a flow controller (MKS Instruments, Munich, Germany). Temperature measurement (calorimetric measurement) showed 42°C at the tip of the plasma jet. We had a constant pin-to-disc distance of 7 mm during the application. The plasma treatment was named ''Ar plasma'' if no oxygen was admixed to the discharge and ''Ar+1% O_2 plasma'' if 1.0% (0.05 slm) oxygen was used as admixture, respectively. In a plasma jet a high gas stream is necessary to cool down the plasma (Bender et al. 2010).

Dielectric barrier discharge (DBD)

Besides the jet we used two different DBD devices. Here, one of the two metal electrodes is covered with a dielectric layer. Due to the existence of the dielectric barrier and an inherent wall charge mechanism the transition

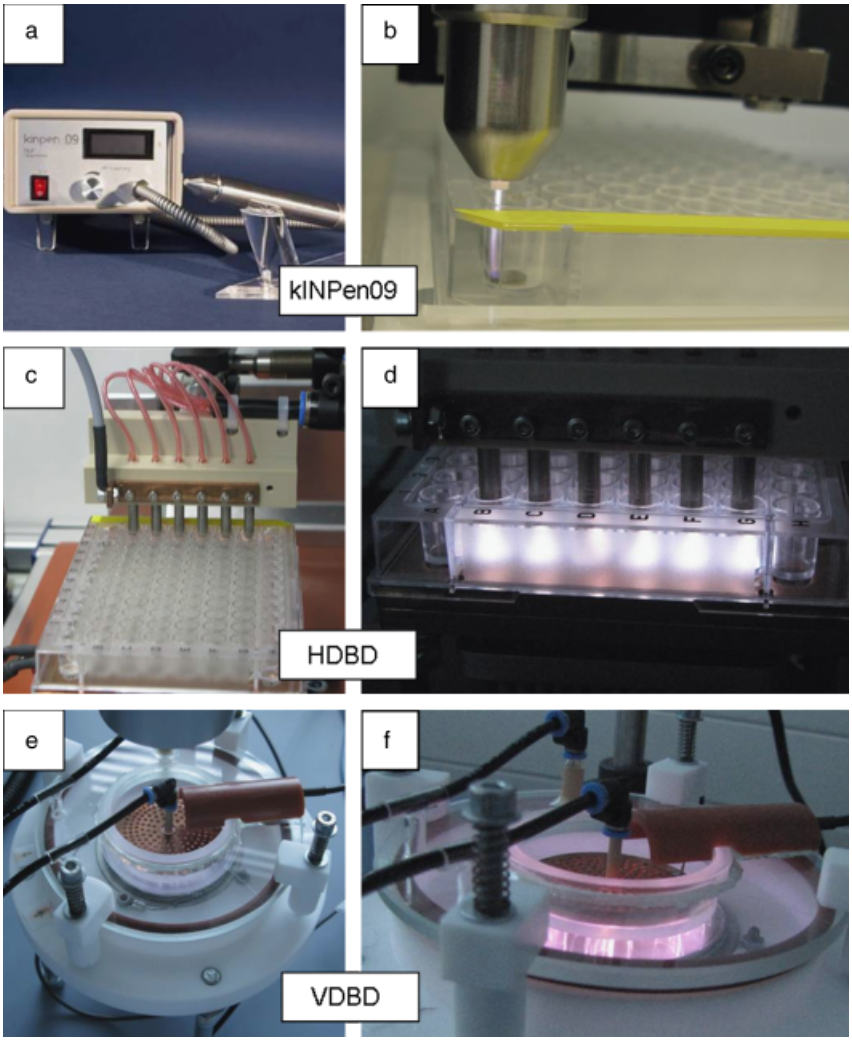

Fig. 1. Photographs of the different plasma devices used in this study. (a+b) kINPen09, (c+d) hollow electrodes dielectric barrier discharge (HDBD), and (e+f) volume dielectric barrier discharge (VDBD).

to an arc discharge is prevented. As a result, the discharge creates non-thermal plasma without substantial heating of the gas. Thus, DBD plasma processes work with a low gas flow.

Hollow DBD electrode (HDBD)

The HDBD (see Fig. 1c,d) has originally been developed for the treatment of samples placed in wells of microtitre plates. Because microtitre plates consist of a dielectric material, they can serve as barrier in a DBD arrangement. These are placed on the grounded electrode, which was cooled down by a Peltier-element to control the temperature of the objects during plasma treatment. Six hollow and thin metal tubes (outer diameter 4 mm; inner diameter 2 mm) served both as high voltage electrodes and gas injection pipes (tube–disc distance 5 mm). The gas flew through these electrodes, while a high RF-voltage (37.6 kHz, 8.4 kV) was coupled. Ar gas flow was set to 1 slm per well. If oxygen was admixed, the total oxygen flow was set to 0.01 slm.

KINPen09 and HDBD were fixed in a computer driven 3-axes (x, y, z) motorized stage, under which a microtitre plate with the titanium discs was positioned. The plasma devices were consecutively driven from well to well, positioned centrally over the discs and remained in position for the respective treatment time.

VDBD

The VDBD consisted of two flat round metal electrodes with one of them being electrically grounded (Fig. 1e,f). A Petri dish (22.1 cm^2 Techno Plastic Products AG, Trasadingen, Switzerland) with the titanium discs was located between these electrodes. The bottom of the Petri dish acted as the dielectric for the DBD. For cooling a Peltier-element was used. The distance between disc and electrode was 15 mm and sealed air-tight. Ar gas (0.05 slm) flew into and out of the system via hoses. The high sinusoidal voltage (40 kHz, 10 kV) applied between both electrodes generated the plasma. Eight discs were treated simultaneously.

There were two adjustable parameters for plasma treatment of titanium discs: (i) the time interval (1, 2, 5, and 10 min) and (ii) the plasma gas composition. Two different gas compositions of plasma (Ar plasma and Ar+1% O_2 plasma; in the case of VDBD only Ar plasma) were applied for 1, 2, 5, or 10 min to the discs with the *S. mutans* or the saliva biofilm. We repeated the treatment procedure eight times. Altogether 320 discs were treated with three plasma devices [two gas admixtures × four treatment intervals × eight repetitions in × two biofilm models × two plasma devices (kINPen09 and HDBD); one gas admixture × four treatment intervals × eight repetitions × two biofilm models in the case of VDBD].

To assess the effects of biofilm dehydration by gas flow (negative control), eight discs were treated with gas (Ar or Ar+1% O_2) and as positive control eight discs were treated with CHX for each treatment time. As a negative control for CHX treatment we used 100 μl 0.9% NaCl solution (called NaCl control, $n = 8$). So we used altogether 352 control discs.

Scanning electron microscopy (SEM)

For electron microscopy the titanium discs of saliva biofilms were prepared as follows: after a fixation step [1 h in 1% glutaraldehyde, 2% paraformaldehyde, 0.2% picric acid, 5 mM HEPES (pH 7.4), and 50 mM NaN_3], the samples were treated with 2% tannic acid for 1 h, 1% osmium tetroxide for 1 h, 1% thiocarbohydrazide for 30 min, 1% osmium tetroxide at 4°C overnight, and with 2% uranyl acetate for 2 h with washing steps in between. Samples were dehydrated in a graded series of acetone solutions (10–100%) and then critical point dried.

Statistics

Continuous data are presented as mean standard deviation.

Table 1. Logarithm of *Streptococcus mutans* biofilm colony forming units (CFU/ml) after treatment with different plasma devices combined with argon (Ar) gas, Ar plasma, Ar+1%O_2 gas, or Ar+1%O_2 plasma for varying exposure times in comparison to untreated controls and CHX treated samples (negative controls)

	Maximum RF	Exposure time (min)				
		0	1	2	5	10
Control		8.35 ± 0.08				
CHX	3.36		5.33 ± 0.12*	5.62 ± 0.23*	5.36 ± 0.10*	4.99 ± 0.18*
kINPen09						
Ar gas	2.05		7.06 ± 0.19*	7.29 ± 0.33*	6.30 ± 0.15*	6.40 ± 0.25*
Ar plasma	3.19		5.16 ± 0.31*	5.48 ± 0.19*	5.32 ± 0.29*	5.32 ± 0.38*
Ar+1%O_2 gas	2.67		6.94 ± 0.21*	6.24 ± 0.13*	6.15 ± 0.12*	5.68 ± 0.19*
Ar+1%O_2 plasma	2.21		6.14 ± 0.22*	6.40 ± 0.31*	6.17 ± 0.35*	6.46 ± 0.55*
HDBD						
Ar gas	1.56		7.86 ± 0.27	7.78 ± 0.18*	7.31 ± 0.23*	6.97 ± 0.11*
Ar plasma	1.79		7.34 ± 0.15*	7.06 ± 0.15*	6.60 ± 0.24*	6.56 ± 0.41*
Ar+1%O_2 gas	1.21		8.36 ± 0.13	8.53 ± 0.17	7.87 ± 0.26*	7.14 ± 0.24*
Ar+1%O_2 plasma	3.79		8.35 ± 0.16	7.43 ± 0.35*	5.32 ± 0.45*	4.56 ± 0.29*
VDBD						
Ar gas	2.74		7.29 ± 0.12*	7.59 ± 0.11*	6.88 ± 0.20*	5.61 ± 0.40*
Ar plasma	5.38		6.28 ± 0.07*	5.01 ± 0.56*	3.20 ± 0.52 *#	2.97 ± 0.41 *#

*$p < 0.05$ *versus* control (0 min), one-sided Mann–Whitney *U*-test.
#$p < 0.05$ *versus* CHX within the same exposure times, one-sided Mann–Whitney *U*-test.
Mean CFU ± SD.
Max RF, maximum reduction factor; CHX, chlorhexidine digluconate; HDBD, hollow dielectric barrier discharge; VDBD, volume dielectric barrier discharge.

First, CFU values were compared for each procedure and exposure time *versus* the NaCl control using Mann–Whitney *U*-tests. Second, CFU values were compared for each procedure (kINPen09, HDBD, and VDBD combined with gas/plasma compositions) *versus* CHX within varying exposure times using one-sided Mann–Whitney *U*-tests. For each step, *p* values were corrected for multiple testing according to Benjamini–Hochberg (Benjamini & Hochberg 1995). Treatment procedures (kINPen09, HDBD, or VDBD with gas/plasma compositions) which did not reduce CFU values significantly compared with the control or CHX were excluded from further analyses.

Then, analyses of variance (ANOVA) and multivariable linear regression analyses were applied to evaluate differences in CFU values for different plasma devices (HDBD combined with Ar plasma+1% O_2, VDBD combined with Ar gas and Ar plasma) and treatment time (1, 2, 5, or 10 min). For twofold interaction terms between both factors a less conservative *p* value of $p < 0.10$ was considered as statistically significant. Finally, within each exposure time, VDBD combined with Ar plasma was compared against the other two devices applying post hoc Wald tests for linear hypotheses.

Statistical significance was declared as $p < 0.05$, 0.01, or 0.001, as appropriate. Statistical analyses were performed with STATA/SE 10.0 (Stata Corp LP, College Station, TX, USA) and R (free shareware, http://www.r-project.org).

Results

S. mutans biofilm treatment

First, CFU values were compared for each procedure and exposure time *versus* 0.9% NaCl solution controls. Except four HDBD procedures (HDBD Ar+1% O_2 gas for 1 and 2 min and HDBD Ar+1% O_2 plasma, 1 min), all other procedures revealed better anti-*S. mutans* effects than 0.9% NaCl ($p < 0.05$) (Table 1).

Second, kINPen09, HDBD, and VDBD procedures were compared against CHX within exposure times. Only for VDBD with Ar plasma (5 and 10 min) CFUs were significantly reduced compared with CHX ($p < 0.05$). For kINPen09, HDBD, and VDBD with Ar gas, CFU values were not consistently reduced compared with CHX (Table 1).

Additionally we determined maximum RF. *S. mutans* was very sensitive to CHX (RF = 3.36, after 10 min), while kINPen09 plasma achieved a maximum RF of 3.19 (after 1 min Ar plasma) and 2.21 (after 1 min Ar+1% O_2 plasma). Corresponding RFs for Ar gas were 2.05 and 2.67. Maximum RFs for DBD were consistently achieved after 10 min. HDBD Ar+1% O_2 plasma achieved a maximum RF of 3.79 whereby VDBD Ar plasma reduced the *S. mutans* biofilm by 5.38 $\log_{10}$.

ANOVAs and linear regression models were used to evaluate the impact of gas composition and exposure time on CFU values (Table 2). The ANOVA explained 79.2% of the variation in observed CFU values. For exposure times of 5 and 10 min. CFUs were significantly reduced for HDBD Ar+1% O_2 plasma ($p < 0.001$) compared with 1 min exposure (see also Fig. 1). Increased exposure times (i.e. 2, 5, or 10 min) and the use of VDBD with Ar gas or Ar plasma further reduced CFU values. Post hoc analysis revealed that VDBD Ar plasma performed significantly best compared with HDBD Ar+1% O_2 plasma and VDBD Ar gas within exposure times ($p < 0.05$, Fig. 2a).

Saliva biofilm treatment

Treatment with kINPen09 combined with Ar plasma, most treatments with HDBD, and all treatments with VDBD (both compositions, all exposure times) showed a better anti-biofilm effect than the 0.9% NaCl solution control at the various exposure times ($p < 0.05$) (Table 3). Compared with CHX within exposure times, only treatment with kINPen09 with Ar plasma (1 and 2 min), HDBD with Ar+1% O_2 plasma (5 and 10 min), and VDBD with Ar gas (1, 2, and 10 min) or Ar plasma (all exposure times) achieved a significant reduction in CFUs ($p < 0.05$, Table 3).

Partly, CFU values were even higher after treatment with kINPen09, HDBD, or VDBD combined with Ar gas compared with CHX-treated samples (Table 3). Thus, kINPen09 and HDBD (except combination with Ar+1% O_2 plasma) were omitted from further analyses.

After treatment of saliva biofilms, CHX achieved a maximum RF of 1.50 $\log_{10}$, which was similar to that of kINPen09 Ar plasma (maximum RF = 1.88). Gas reduced CFUs only slightly (kINPen09: $RF_{Ar\ gas} = 0.42$, $RF_{Ar+1\%\ O_2\ gas} = 0.78$; HDBD: $RF_{Ar\ gas} = 1.39$, $RF_{Ar+1\%\ O_2\ gas} = 0.88$; VDBD: $RF_{Ar\ gas} = 2.08$). VDBD Ar plasma achieved the highest maximum RF (RF = 5.67).

ANOVAs and linear regression models were used to evaluate the impact of gas composition, exposure time, and the interaction of both on saliva CFU values (Table 4). The ANOVA model explained 82.5% of the variation in observed CFU values. After 2, 5, and 10 min, CFUs were significantly reduced for HDBD Ar+1% O_2 plasma (−0.91, −2.67, and −1.77, respectively). Compared with HDBD Ar+1% O_2 plasma, use of VDBD with Ar gas or Ar plasma further reduced CFU values significantly ($p < 0.001$). However, reduction in CFU values for increasing exposure times was less pronounced for VDBD Ar gas compared with HDBD with Ar+1% O_2 plasma (see Fig. 2b). For 5 and 10 min of exposure, predicted CFU values were even slightly higher for VDBD with Ar gas compared with HDBD combined with plasma+1% O_2. The time dependent decrease in CFU values was most obvious for VDBD with Ar plasma (Fig. 2b). After 10 min, VDBD with Ar plasma achieved the highest reduction in CFUs with predicted log CFUs being −5.38 (95%CI: −6.39; −4.37) lower compared with HDBD Ar+1% O_2 plasma (1 min).

Post hoc analyses revealed that VDBD Ar plasma performed significantly best compared with HDBD Ar+1% O_2 plasma and VDBD Ar gas within exposure times ($p < 0.001$, Fig. 2b).

Table 2. ANOVA and according linear regression models evaluating effects of method [VDBD combined with argon (Ar) gas or Ar plasma] and exposure time on *Streptococcus mutans* CFUs

	ANOVA	Linear regression	
	p value	*B* (95% CI)	*p* value
Time (ref.: 1 min)			
2 min		−0.92 (−2.00; 0.16)	0.09
5 min		−3.03 (−4.11; −1.95)	<0.001
10 min	<0.001	−3.80 (−4.88; −2.72)	<0.001
Method (ref.: HDBD Ar+1%O_2 plasma)			
VDBD Ar gas		−1.06 (−2.08; −0.05)	0.04
VDBD Ar plasma	<0.001	−2.08 (−3.16; −1.00)	<0.001
Method × time			
VDBD Ar gas, 2 min		1.23 (−0.20; 2.66)	0.09
VDBD Ar gas, 5 min		2.63 (1.18; 4.08)	0.001
VDBD Ar gas, 10 min		2.11 (0.69; 3.54)	0.004
VDBD Ar plasma, 2 min		−0.35 (−1.84; 1.15)	0.65
VDBD Ar plasma, 5 min		−0.05 (−1.53; 1.43)	0.95
VDBD Ar plasma, 10 min	0.004	0.49 (−0.99; 1.97)	0.51
constant		8.35 (7.59; 9.12)	<0.001

$R^2 = 79.2\%$.

HDBD, hollow dielectric barrier discharge; VDBD, volume dielectric barrier discharge.

Comparison of *S. mutans* and saliva biofilm reduction

Because initial concentrations of *S. mutans* and saliva were similar (Tables 1 and 3), we compared CFU values for both biofilms after treatment using two-sided Mann–Whitney *U*-tests and adjusting *p* values for multiple testing. Except three kINPen09 procedures (Ar gas and Ar plasma for 2 min and Ar+1% O_2 plasma, 10 min), kINPen09 was

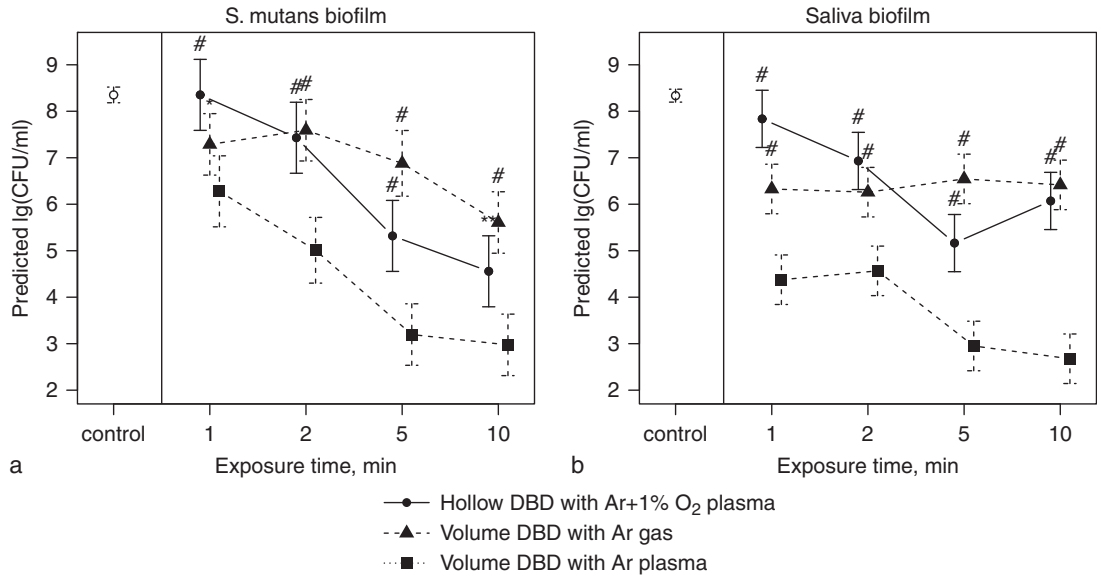

Fig. 2. Predicted *Streptococcus mutans* (a) or saliva (b) biofilm CFU (mean with 95% CI) after treatment with hollow electrodes dielectric barrier discharge (HDBD) with Ar+1%O_2 plasma (circles) and volume dielectric barrier discharge (VDBD) with Ar gas (triangles) and Ar plasma (rectangles) for different exposure times. Observed CFU for negative controls are additionally given. $^{*}p < 0.05$, $^{**}p < 0.01$, # $p < 0.001$ *versus* VDBD with Ar plasma within exposure times applying post hoc Wald tests.

Table 3. Logarithm of saliva biofilm colony forming units/titanium discs after treatment with different plasma devices combined with argon (Ar) gas, Ar plasma, Ar+1%O_2 gas, or Ar+1%O_2 plasma for varying exposure times in comparison with untreated controls and CHX treated samples (negative controls)

	Max RF	Exposure time (min)				
		0	1	2	5	10
Control		8.34 ± 0.07				
CHX	1.50		7.42 ± 0.23	7.07 ± 0.15	6.84 ± 0.15	7.16 ± 0.23
kINPen09						
Ar gas	0.42		7.98 ± 0.12	7.92 ± 0.17	7.92 ± 0.17	7.97 ± 0.11
Ar plasma	1.88		6.65 ± 0.18*#	6.46 ± 0.34*#	6.81 ± 0.22*	6.92 ± 0.11*
Ar+1%O_2 gas	0.60		7.93 ± 0.18	8.03 ± 0.10	7.82 ± 0.20	7.74 ± 0.14
Ar+1%O_2 plasma	0.78		7.96 ± 0.04	7.79 ± 0.12	7.86 ± 0.05	7.56 ± 0.07
HDBD						
Ar gas	1.39		7.28 ± 0.09*	7.08 ± 0.08	7.38 ± 0.03	6.95 ± 0.12*
Ar plasma	1.65		7.07 ± 0.14*	6.69 ± 0.14*	6.88 ± 0.06	6.77 ± 0.09*
Ar+1%O_2 gas	0.88		8.45 ± 0.11	8.29 ± 0.14	7.58 ± 0.05	7.46 ± 0.09
Ar+1%O_2 plasma	3.18		7.84 ± 0.08	6.93 ± 0.24*	5.16 ± 0.10*#	6.07 ± 0.14*#
VDBD						
Ar gas	2.08		6.33 ± 0.16*#	6.26 ± 0.14*#	6.54 ± 0.13*	6.42 ± 0.22*#
Ar plasma	5.67		4.38 ± 0.29*#	4.57 ± 0.32*#	2.95 ± 0.52*#	2.67 ± 0.40*#

*$p<0.05$ *versus* control (0 min), one-sided Mann–Whitney *U*-test.
#$p<0.05$ *versus* CHX within the same exposure times, one-sided Mann–Whitney *U*-test.
Max RF, maximum reduction factor; CHX, chlorhexidine digluconate; HDBD, hollow dielectric barrier discharge; VDBD, volume dielectric barrier discharge. Mean CFU ± SD.

Table 4. ANOVA and according linear regression models evaluating effects of method [HDBD combined with argon (Ar)+1% O_2 plasma and VDBD combined with Ar gas or Ar plasma] and exposure time on saliva CFUs

	Linear regression		ANOVA
	B (95% CI)	*p* value	*p* value
Time (ref.: 1 min)			
2 min	−0.91 (−1.78; −0.04)	0.042	
5 min	−2.67 (−3.54; −1.80)	<0.001	
10 min	−1.77 (−2.64; −0.90)	<0.001	<0.001
Method (ref.: HDBD Ar+1%O_2 plasma)			
VDBD Ar gas	−1.51 (−2.32; −0.69)	<0.001	
VDBD Ar plasma	−3.46 (−4.27; −2.65)	<0.001	<0.001
Method × time			
VDBD Ar gas, 2 min	0.84 (−0.31; 1.99)	0.15	
VDBD Ar gas, 5 min	2.89 (1.74; 4.04)	<0.001	
VDBD Ar gas, 10 min	1.85 (0.70; 3.01)	0.002	
VDBD Ar plasma, 2 min	1.10 (−0.06; 2.25)	0.062	
VDBD Ar plasma, 5 min	1.25 (0.10; 2.40)	0.034	
VDBD Ar plasma, 10 min	0.07 (−1.09; 1.22)	0.91	<0.001
constant	7.84 (7.22; 8.45)	<0.001	

$R^2 = 82.5\%$
HDBD, hollow dielectric barrier discharge; VDBD, volume dielectric barrier discharge.

significantly more efficient against *S. mutans* biofilms than against saliva biofilms. Results were consistent for HDBD Ar+1% O_2 plasma (1 and 10 min), HDBD Ar gas (2 min), VDBD Ar gas (1 and 2 min) and VDBD Ar plasma (1 min).

Microscopy

SEM micrographs of 10 min plasma-treated cells revealed massive perforations of cell walls (Fig. 3). Most cells were completely destroyed by the plasma jet. SEM micrographs of 10 min kINPen09-treated cells were similar to those of HDBD and VDBD (Fig. 3d). In contrast, CHX-treated cells looked as undamaged as the 0.9% NaCl solution controls. They seemed to be covered by a layer (Fig. 3b).

Discussion

Experimental settings

Implant failure has classically been attributed to bacterial infection (Berglundh et al. 2002). The aetiology of peri-implantitis and peri-implant mucositis is thought to be an infection (Isidor 1997, Sennerby et al. 2005). Untreated bacterial infection leads to inflammatory peri-implant disease. Peri-implant mucositis is a reversible inflammatory soft tissue lesion in contrast to peri-implantitis with irreversible bone loss around the implant (Kivela-Rajamaki et al. 2003). To prevent the progression from a peri-mucosal to peri-implant lesion, dental treatment should already be delivered in the state of peri-implant mucositis.

Numerous treatment regimens of peri-implant inflammatory lesions have been recommended. A pathology stage dependant protocol of therapeutic measures has been described (Cumulative Interceptive Supportive Therapy), where the antiseptic use of a 0.1–0.2% chlorhexidine solution is a central feature of the peri-mucosal treatment (Lang et al. 2000). In a previous study, 2% CHX was used to obtain an effect upon established plaque (Löe & Schiott 1970). However, because currently applied concentrations of 0.1–0.2% CHX caused staining of teeth and tongue and dysgeusia in patients (Moshrefi 2002, Gurgan et al. 2006), a 2% chlorhexidine concentration would be too high and implausible for clinical usage. Furthermore, hyperceratosis, ulceration, dysplasia, and a significant increase of DNA damages were observed in rat experiments (Sonis et al. 1978, Grassi et al. 2007). Moreover it cannot be

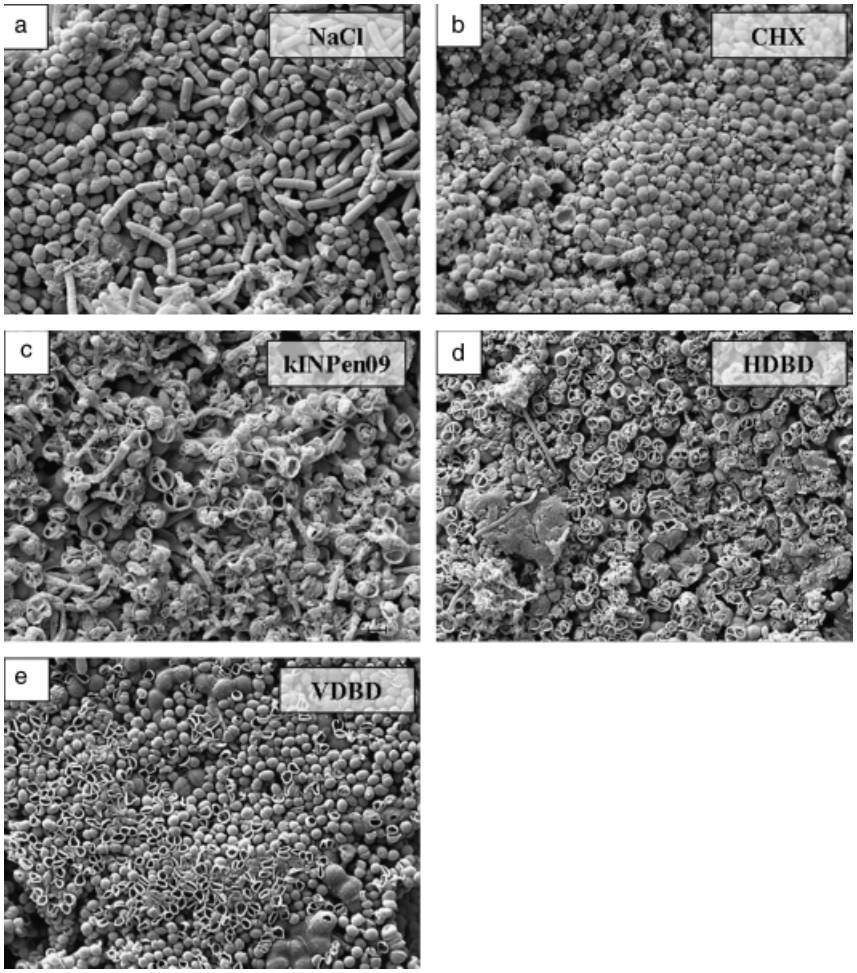

Fig. 3. SEM micrographs of saliva biofilms (a–e) on titanium discs after 10 min treatment with (a) 0.9% NaCl solution (negative control), (b) chlorhexidine digluconate, (c) with kINPen09, (d) with hollow electrode dielectric barrier discharge (HDBD), and (e) with volume dielectric barrier discharge (VDBD). Magnification was 5.000-fold.

assumed that a high concentration in vivo corresponds to a high concentration in vitro, due to leaching by crevicular fluid and saliva in vivo. In our in vitro experiments leaching was eliminated because biofilms were directly covered with CHX without any diluting effects. Therefore we used 0.1% CHX solution as a standard concentration for these tests.

For plaque removal from the abutment, metallic curettes or sonic or ultrasonic inserts are not advocated because they damage and roughen the abutment and thus facilitate plaque retention (Quirynen et al. 1993). Another mechanic treatment option are plastic coated sonic or ultrasonic inserts, which are, however, worn off at the abutment and leave plastic contamination and residues (Rühling et al. 1994). It is unknown whether these residues impact the resolution of the inflammatory lesion. Therefore, effective and predictable antimicrobial treatment regimes that reduce or remove the biofilm without damaging abutment surface are of great interest.

In this study, anti-biofilm effects of different plasma devices were compared with those of CHX as an antiseptic, which usually has a concentration of 0.2% for peri-implant mucositis therapy (Lang et al. 2000). Our CHX results are in accordance with the literature in that a 0.1% CHX solution is inefficient against established oral biofilms (Vitkov et al. 2005). Therefore, alternative methods are warranted. In our experiments, we demonstrated that two different plasma devices were significantly more effective in the treatment of dental biofilms compared with CHX.

S. mutans biofilm

The *S. mutans* biofilm was more sensitive than the saliva biofilm against all antimicrobial agents used (CHX, plasma, and oxygen gas). For treatment with kINPen09 we observed the highest $\log_{10}$ RF (RF = 3.19) after 1 min; a short treatment time seemed to be sufficient. For both DBD sources there were time dependant treatment effects and the highest CFU reductions were observed after 10 min. Besides the exposure time the gas mixture also influenced the effect of different plasma devices. Admixture of 1% O_2 gas (without plasma) resulted in higher CFU reductions compared with treatment with pure Ar gas, probably due to oxidative stress. Only VDBD plasma showed significantly higher RF than CHX. CHX had a high anti-microbial effect (3.36 $\log_{10}$) in this monospecies biofilm model, which is in agree with in vitro and in vivo results (Maltz et al. 1981, Jarvinen et al. 1993). Our results clearly indicate that *S. mutans* monospecies biofilms do not present a suitable model for biofilms on dental implants.

Other in vitro studies reported an antimicrobial effect of plasma against *S. mutans* grown on agar plates as well as against adherent bacteria and 24 h *S. mutans* biofilms (Goree et al. 2006, Sladek et al. 2007, Rupf et al. 2010). Monospecies biofilms like immature *E. coli* and *Staphylococcus* biofilms were successively removed by plasma (Lee et al. 2009). We observed similar results with *Candida albicans* biofilms with an RF of 5 $\log_{10}$ steps (Koban et al. 2010). All these plasma biofilm studies used different plasma sources – a direct comparison is not possible. Importantly, no other study achieved an RF above 5 $\log_{10}$ steps.

Saliva biofilm

However, both peri-implantitis and peri-implant mucositis are not infections by single pathogens; rather, they are multimicrobial infections. Therefore, we used saliva biofilms. Because the in vivo plaque is highly diverse and complex (Moore & Moore 1994), biofilms containing multiple pathogenic species are more relevant for studying dental diseases (Shu et al. 2000). Microorganisms in a multispecies biofilm are better protected because the whole biofilm and especially the extracellular matrix is more complex. Simplified in vitro biofilm models may help to clarify the effectiveness of antimicrobial treatment strategies under standardized laboratory conditions (Müller et al. 2007). Thus,

our multispecies biofilm presents a more realistic model of an oral biofilm than a monospecies biofilm and may be a better choice for testing antimicrobial influences of non-thermal plasma.

On saliva biofilms kINPen09 (1 and 2 min), HDBD Ar+1% O_2 (5 and 10 min), and VDBD plasma (5 and 10 min) had significantly greater antimicrobial effects compared with CHX. VDBD Ar plasma achieved the greatest reduction of 5.67 CFUs, whereas CHX reduced saliva biofilm about 1.5 CFUs. Possibly, VDBD related techniques might be promising.

In contrast to the *S. mutans* biofilm, admixture of 1% O_2 gas (without plasma) did not result in higher CFU reductions on saliva biofilms compared with treatment with pure Ar gas. Possibly, the saliva biofilm might be more resistant to oxidative gases than the monospecies *S. mutans* biofilm. Nevertheless, the RF for HDBD plasma was doubled using the admixture of O_2 (RF = 3.18) compared with pure Ar plasma (RF = 1.65), because of produced reactive oxygen species inducing oxidative stress. In the gas phase of plasma processes, atomic oxygen O and hydroxyl OH radicals are produced by electron-impact dissociation of air molecules (Goree et al. 2006). Thus, anaerobes might be more sensitive to plasma and the RF of anaerobic biofilms might be even better than the RF of the saliva biofilm. HDBD seems to generate more of these species than kINPen09 because with this plasma jet we could not find any additional effect of oxygen admixture. At the time of our experiments, VDBD trials with O_2 admixture were not possible. Experiments with this setting should be performed in future if we can modify our VDBD device to be used with an admixture of O_2.

Comparison of operation modes for different plasma devices

SEM micrographs demonstrated an effect in a circumscribed spot for the kINPen09 treatment (Fig. 1b). In this area, the shape of destroyed cells was comparable to DBD-treated cells. Nevertheless, total CFU values were less reduced with kINPen09 compared with DBD. One reason for this lower antimicrobial effect of the kINPen09 could be that the treatment with the narrow plasma effluent damages only cells at the zone where the effluent touches the disc. Reactive plasma species were probably not spread over the entire disc in a sufficient dose. In further experiments, the pen should be continuously moved over the disc instead of treating only one single spot. The maximal effect of the pen was already reached after 1 min. It may be that a layer of dead superficial microbiota sheltered the living bacteria underneath when we extended the treatment time.

DBD plasma consists of many microlightning, which are clearly visible in Fig. 1f and these last only few nanoseconds (Liu et al. 2004). With the DBD plasma the whole disc was treated in contrast to spot restricted kINPen09 treatment. The disadvantage of DBD plasma is that it is not homogenous in comparison to kINPen09. Thus, some regions remained undamaged probably due to the inhomogeneities of DBD plasma. Apparently intact cells could be observed between damaged cells (Fig. 3e).

DBD plasma devices are subdivided into HDBD and VDBD devices. VDBD plasma with an exposure time of 10 min showed the highest reduction rate in both biofilms ($RF_{S.\ mutans} = 5.38$; $RF_{saliva} = 5.67$), though the difference between the *S. mutans* and the saliva biofilm was not statistically different. While the VDBD worked with an input power of 16 W, 40 kHz, and 10 kV, the HDBD worked with an input power of 9 W, 37.6 mHz, and 9 kV. Due to different input power settings a direct comparison of both plasmas is difficult. In order to understand if the higher power setting of VDBD and/or the different plasma species due to the different construction are responsible for these results, further plasma diagnostic investigations by physicists are necessary.

Study limitations

Many simple monobacterial biofilm models have been developed, e.g. using *S. mutans* (Noorda et al. 1986a, b, Honraet et al. 2005, Sladek et al. 2007, Pasquantonio et al. 2008). Because of its easy growth requirements we used *S. mutans* as a standardized biofilm model to screen the effects of the different plasma devices. Though *S. mutans* biofilms have the advantage of being standardized, they are much more sensitive to antimicrobial manipulation than saliva biofilms. After the initial screening stage we applied a saliva biofilm, which may a little bit more reflect the clinical reality in a peri-mucosal pocket.

Our study had the limitation that we used aerobic cultivation methods and furthermore a 2-day-old in vitro biofilm. In an anaerobic biofilm model a selective suppression of Gram-negative anaerobic species occurs after plasma treatment (Filoche et al. 2008). However, in peri-implant diseases aerobic pathogens like Pseudomonas spp., staphylococci, enterics and Candida were more often found than in healthy implant situations (Leonhardt et al. 2003, Botero et al. 2005). Even if the aerobic biofilm is not the first target of plasma application, its treatment gives insight into possible plasma actions. Our next experimental step will include anaerobic biofilms and biofilms grown intraorally on discs (Meyerowitz, 1991 p. 467; Sedlacek, 2007 p. 182).

Another limitation concerns the plasma devices themselves. Both DBD devices are spacious and big. The physicists and engineers constructed them to test different plasma parameters in vitro and to understand the principles of cold plasma generation. The DBD devices used here were not intended to be used as a medical device. Our results should be just understood as a proof of principle. If we want to continue their possible application in dentistry, our devices have to reengineered and miniaturized for animal and patient experiments. Moreover, a 10-min treatment period would not be feasible in dental practice. Nevertheless, we can state from these pilot trials that plasma is an effective antimicrobial agent and further technical development of plasma sources might enable clinical utilization.

Biofilms play a major role in the pathogenesis of various oral diseases, especially peri-implant mucositis. In this study, we investigated the effect of three different plasma devices on *S. mutans* and multispecies saliva biofilms on titanium discs in comparison to CHX in vitro. In contrast to CHX, treatment with non-thermal plasma was very effective against *S. mutans* and multispecies saliva biofilms. Future research will elucidate the cause of the different efficacies of these plasma devices and we will try to develop devices and methods, which can be used for biofilm-driven infections in the dental practice.

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Address:
Ina Koban
Department of Restorative Dentistry, Periodontology, and Endodontology
Unit of Periodontology
Ernst-Moritz-Arndt University Greifswald
Walther-Rathenau-Straße 49a
17487 Greifswald
Germany
Email: ina.koban@uni-greifswald.de

Clinical Relevance

Scientific rationale for the study: The antimicrobial efficacy of non-thermal plasma was assessed with a monospecies *S. mutans* and a multispecies saliva biofilm model.

Principal findings: Plasma, especially DBD plasma, was significantly more effective against monospecies and multispecies biofilms compared to CHX.

Practical implications: Once the plasma devices used in this study are further developed for clinical use, plasma might be an effective treatment option in peri-implant mucositis.