J Clin Periodontol 2015; 42: 478–487 doi: 10.1111/jcpe.12403

Journal of Clinical Periodontology

Effects of non-equilibrium plasma in the treatment of ligature-induced peri-implantitis

Qi Shi[1,*], Ke Song[1,*], Xincai Zhou[2], Zilan Xiong[3], Tianfeng Du[1], Xinpei Lu[3] and Yingguang Cao[1]

[1]Department of Stomatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2]Department of Stomatology, Shenzhen Baoan Maternal and Child Health Hospital, Shenzhen, China, [3]State Key Laboratory of Advanced Electromagnetic Engineering and Technology, Huazhong University of Science and Technology, Wuhan, China

*These authors are contributed equally to this work.

Shi Q, Song K, Zhou X, Xiong Z, Du T, Lu X, Cao Y. Effects of non-equilibrium plasma in the treatment of ligature-induced peri-implantitis. J Clin Periodontol 2015; 42: 478–487. doi: 10.1111/jcpe.12403.

Abstract

Aim: To evaluate the effects of non-equilibrium plasma in the treatment of ligature-induced peri-implantitis in beagle dogs.

Materials and Methods: Six beagles received 12 implants installed in the position of the fourth mandibular premolars. Ligature-induced peri-implantitis was initiated at 3 months post-implantation. When approximately 40% of the supporting bone was lost, the ligatures were removed. The implants were subjected to the muco-periosteal scaling and chlorhexidine irrigation with or without plasma irrigation. Three months later, clinical, radiographic and microbiological analyses were performed. Block biopsies were prepared for micro-CT and histomorphometric analysis. The primary outcome was the difference in bone healing of peri-implant sites, and the secondary outcomes included changes in clinical parameters (SBI, PD) and bacterial detection.

Results: At baseline, no significant differences were observed between the two groups. At 3 months post-treatment, the plasma group showed a significantly higher bone level than the control group ($p < 0.05$), a significantly decreased detection of bacteria (*Porphyromonas gingivalis* and *Tannerella forsythia*) ($p < 0.05$), and a significant improvement in clinical examination ($p < 0.05$).

Conclusions: Within the limits of this study, non-equilibrium plasma treatment as an adjunct to the conventional therapy is a feasible approach for the treatment of peri-implantitis.

Key words: animal experiment; dental implants; infections; non-equilibrium plasma; peri-implantitis

Accepted for publication 14 July 2014

Introduction

Peri-implantitis is an inflammatory disease that affects peri-implant soft and hard tissues, which is characterized by bleeding on probing and progressive crestal bone loss (Lang & Berglundh 2011). If left untreated, it may even cause progressive increased implant mobility and eventual implant loss (Heitz-Mayfield 2008). While dental implant installation has a high success rate, it is associated with a peri-implantitis rate of 10.7–47.2% within 10 years after implantation (de Waal et al. 2012).

Bacterial biofilms play a key role in the onset and progression of peri-implantitis lesions (Lindhe et al. 1992, Mombelli & Lang 1998); therefore, biofilm removal is important for the treatment of peri-implantitis. Currently, different treatment strategies (surgical and non-surgical methods such as mechanical debridement,

Conflict of interest and Source of Funding Statement
The authors declare that they have no conflict of interest.
This work was supported by grants from the National Natural Science Foundation of China (No. 81271189), the Specialized Research Foundation for the Doctoral Program of Higher Education of China (No. 20120142110054) and the Fundamental Research Funds for the Central Universities (No. 2010JC034).

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

chemical antiseptics, laser, etc.) for peri-implantitis have been suggested and have proven to be effective. However, there is no reliable evidence that suggests which interventional treatment may be the most efficacious (Esposito et al. 2012). Moreover, implant surfaces are known to be contaminated by some decontamination methods, which results in the smear layer and modified surface topography and ultimately does not permit re-osseointegration (Klinge et al. 2005). Thus, new treatment approaches of peri-implantitis are needed.

Plasma, the fourth state of matter, is an ionized, electrically neutral gas containing numerous active components (including electrons, ions, free radicals and chemically reactive neutral particles; Duske et al. 2012). The plasma system can be thermal or non-thermal. Non-equilibrium plasma is non-thermal plasma, also called cold plasma, which is around room temperature and contains numerous short-lived reactive species (excited OH, N_2, N_2^+, He, O species, etc.) (Lu et al. 2008). Since Laroussi (1995) first showed that non-equilibrium plasma could eradicate living pathogenic microorganisms after exposure for a sufficient time, non-equilibrium plasma has been studied for multiple biological applications, including heat-sensitive medical device disinfection, living tissue decontamination, wound healing and cancer treatment (Halfmann et al. 2007, Isbary et al. 2010, Kim et al. 2011, Heinlin et al. 2013). Non-equilibrium plasma has also been used in some dental experimental studies for bacteria inactivation (Cao et al. 2011), tooth whitening (Kim et al. 2013), dental caries treatment (Sladek et al. 2004) and root canal treatment (Du et al. 2012, Schaudinn et al. 2013), among others. Additionally, non-equilibrium plasma has been shown to be beneficial in inactivating dental biofilms (Koban et al. 2011, Du et al. 2013), disinfecting irregular surfaces such as cracks and fissures (Sladek et al. 2007), rendering surfaces hydrophilic (Vogelsang et al. 2010), improving cell adhesion (Naciri et al. 2014), promoting the healing of dental implants (Koban et al. 2012) and exhibiting synergistic effects with antiseptics (Du et al. 2013). Therefore, non-equilibrium plasma may be an attractive treatment option for peri-implantitis. The findings reported so far, however, are from in vitro studies. To our knowledge, there are only a few studies which investigate destruction of in situ formed oral biofilms from machined as well as micro-structured (SLA) titanium discs via cold plasma (Rupf et al. 2011, Idlibi et al. 2013). There have also been some reports studying whether or not in vitro cold plasma pre-treatment of titanium implants or abutments may improve bone formation in vivo (Canullo et al. 2013, Giro et al. 2013). In this study, we created a new non-equilibrium plasma device based on a plasma instrument mentioned in previous papers (Lu et al. 2009, Cao et al. 2011, Du et al. 2013), and evaluated its effects in the treatment of ligature-induced peri-implantitis in beagles.

Material and Methods

The study protocol was approved by the Ethics Committee of Animal Research of Tongji Hospital of HUST, China. The procedures were performed according to the regional animal welfare requirements and the trans-disciplinary requirements in comparative implant dentistry (Dard 2012). The study was performed from March 2011 to June 2012 and this article has been written following the ARRIVE guidelines (Kilkenny et al. 2011) and the consensus of the eighth European workshop on periodontology (Berglundh and Stavropoulos, 2012).

Animals

Six adult beagles (approximately 18 months old, 12–13 kg) used in this study were purchased from Anlu Ruikesen experimental animals Co., Ltd., Hubei, China (license No. SCXK (Hubei) 2008-0001). None of the animals showed any clinical signs of infection or other oral diseases. During the experiment, the animals were housed separately in kennels of the Experimental Animal Center of Tongji Hospital of HUST, in 100% fresh air, ambient temperature 25 ± 1°C and humidity 40–70%. They were fed a dog diet twice-daily (1–1.5% of body weight), given free access to fresh water and subjected to oral hygiene with a toothbrush and physiological saline every third day.

Any procedure involving animals and their care were performed according to the Prevention of Cruelty to Animals Act 1986, the NIH Guidelines for Care and Use of Laboratory Animals and the local laws.

Non-equilibrium plasma application

Compared to the previously designed devices, there were a few minor differences in this device (Fig. 2a): (i) the medical syringe was replaced by a quartz tube, and the shape and size of the device were similar to those of a dental handpiece; (ii) a plastic needle was installed in front of the handpiece. The needle was removable so that needles of different sizes (length, diameter) could be used as required. The diameters of the handpiece and the plastic needle were 10 mm and 3 mm, respectively. The inner diameter of the needle was approximately 1.5 mm, and the length was 15–20 mm. The HV electrode is powered by a low-frequency radiofrequency supply, operating at 8 kV, 8 kHz, and a pulse width of 1600 ns. The nonequilibrium plasma is generated between the HV electrode and the surrounding air. The visible part of the plasma plume ejected from the plastic needle was approximately 2 cm for peri-implantitis treatment. The working gas used was 0.1% O_2/He; the flow rate of which was controlled by a mass-flow controller set at 2 l/min. The optical emission spectra of the non-equilibrium plasma were shown in Fig. 2b.

Tooth extraction and implant installation

Following a quarantine period of 1 month, the fourth mandibular premolars (PM4) on both sides of each dog were hemisected with a thin fissure bur under copious irrigation and atraumatically extracted with forceps and/or elevators under a general anaesthesia by intravenous administration of pentobarbital sodium (30 mg/kg). The surgical sites were also infiltrated locally with 2% xylocaine with 1:80,000 epinephrine for haemostasis. After 3 months, 12 screw-type titanium implants (ANTHOFIT®; OIIM 35100, Ø 3.5 mm × 10.0 mm length, Anthogyr, French– a BCP® sandblasted

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

surface consisting of a mixture of hydroxyapatite and beta-tricalcium phosphate with Ra ranging from 1.5 to 2.0 μm) (http://www.anthogyr.com/sites/default/files/atoms/files/bcp_brochure.pdf) were placed (two in each dog) under a general anaesthesia as previously described. The shoulders of the implants were flush with the buccal bone crest, and all implants were provided with healing abutments (Ø 4.3 mm × 4.0 mm length, Anthogyr, French). The surgical procedures were performed according to the manufacturer's guidelines. The animals were given carprofen (4 mg/kg, po) daily in the first 3 days after the surgical procedures. The outline of this study is depicted in Fig. 1.

Experimental peri-implantitis

Three months after implantation, experimental peri-implantitis was induced by placing cotton ligatures in a sub-marginal position around the neck of the implants under general anaesthesia, as previously described (Lindhe et al. 1992). The ligatures were replaced once every 3 weeks. The only difference from previous studies was that we used cotton ligatures intertwined with stainless steel ligatures (Ø 0.25 mm); in this way, we reduced the chances of the ligatures from easily falling off the neck portion of implants. Subsequently, the progression of bone loss was observed by clinical and radiological examination. When approximately 40% of the supporting bone was lost, the ligatures were removed.

Treatments

After removing the ligatures, two implants from each dog were randomly assigned to two groups using a randomization software (version 1.0; Random Allocation Software, Isfahan, Iran) and received different treatment under a general anaesthesia (sodium pentobarbital, 30 mg/kg/i.v.).

Control group

The buccal and lingual muco-periosteal flaps were elevated for scaling the implant surface with manual plastic curettes (Hu-Friedy Co. Inc., Chicago, IL, USA), and the visible plaque, calculus and granulation tissues around the implants were curetted. After scaling, all implants were irrigated with 0. 2% chlorhexidine digluconate for 3 min and sterile saline solution for an additional 3 min. The flaps were then replaced and sutured with absorbable 3/0 suture at the level of the implant platform.

Plasma group

This group was subjected to the conventional techniques and non-equilibrium plasma. After the conventional techniques (described above), the plasma was directly irradiated on the buccal, mesial, lingual and distal surfaces of each implant by a scanning method for 3 min. Distance between the plastic needle and implant surface was 5 mm (a schematic diagram is seen in Fig. 2a). All treatments were performed by an experienced operator, who was blinded to the randomization of the study. After treatment, dogs were subjected to regular tooth and implants cleaning with a toothbrush and physiological saline every third day to maintain oral hygiene.

Clinical examination

Clinical examinations were performed at baseline (lig.−), and at month 1, 2 and 3 following ligature removal (Fig. 1). The parameters measured included a sulcus bleeding index (SBI) and probing depth (PD).

Sulcus bleeding index

A manual plastic probe (Merrit B® probe; Hu-Friedy, Chicago, IL, USA) was inserted approximately 1 mm of depth into the peri-implant epithelium, and the sulcus was scratched over its facial and oral surface. Two sites per implant observed for bleeding for 30 s: buccal and lingual, then scores for the two areas are totalled and divided by two. The bleeding index was scored according to the assessment described by Mazza et al. (1981).

Probing depth

The probing depth (PD) was measured from the gingival/mucosal margin to the base of the probe-able pocket at four sites (buccal, mesial, distal and lingual) per implant, using a Merrit B® probe (Hu-Friedy, USA) with a standardized probing force of 0.2 N.

Microbial examination

Microbial samples were obtained from four sites (buccal, mesial, distal and lingual) per implant at baseline, and months 1, 2 and 3 following ligature removal (Fig. 1). If one site is positively tested, the entire implant is positive.

Sampling sites were isolated with sterile cotton rolls. Supragingival plaque was removed with sterile plastic curettes and the sites were gently air-dried. Four paper points (#30) were inserted into the peri-implant pocket until resistance was felt; they were held in place for 30 s. The paper points were then removed and placed into vials containing 0.5 ml sterile phosphate-buffered saline solution and stored at −20°C. The samples were boiled for 10 min, centrifuged, and the supernatant was subsequently used for polymerase

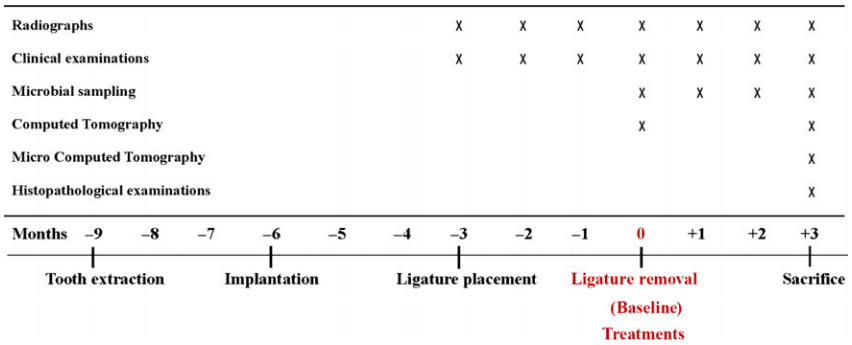

Fig. 1. Study outline: Ligatures were placed 3 months after implant installation (Lig.+) and removed at month 0 (Lig.−, baseline). Animals were treated with different techniques at baseline and sacrificed after 3 months. "X" indicates that the examination was conducted at the corresponding time point.

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

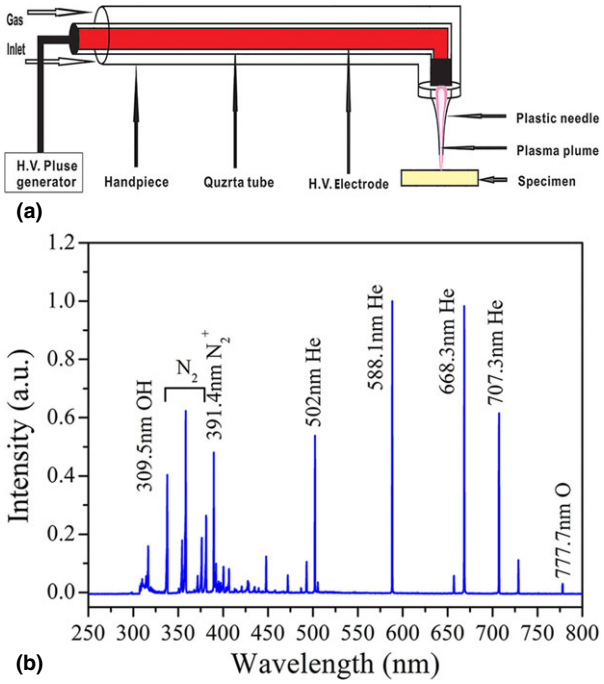

Fig. 2. The non-equilibrium plasma application device. (a) A schematic diagram of the device used. (b) The optical emission spectra of the plasma plume (1% O_2/He-plasma, 8 kV, 10 kHz, 1600 ns). The presence of the short-living excited OH, N_2, N_2^+, He and O species in the plasma plume can be seen.

chain reaction (PCR) analysis. The primer pairs used have been described by Slots et al. (1995): (i) *Porphyromonas gingivalis* (*P. gingivalis*): 5′-AGGCAGCTTGCCATACTGCG-3′ and 5′-ACTGTTAGCAACTACCGATGT-3′, (ii) *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*): 5′-GCTAATACCGCGTAGAGTCGG-3′ and 5′-ATTTCACACCTCACTTAAAGGT-3′, (iii) *Tannerella forsythia* (*T. forsythia*): 5′-GCGTATGTAACCTGCCCGCA-3′ and 5′-TGCTTCAGTGTCAGTTATACCT-3′. PCR amplifications were performed in a reaction mixture containing 5 μl of sample, 1× PCR buffer, 1.0 μM of each primer sample, 200 μM of each of the 4 dNTPs, 1.25 units Taq DNA polymerase, in a final volume of 50 μl. The samples were subjected to a pre-incubation cycle of 5 min at 95°C, incubated for 36 cycles of 30 s at 95°C, 1 min at 60°C and 1 min at 70°C and then a last cycle of 2 min at 72°C. PCR amplification products were analysed by 1.5% agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed under 300 nm an ultraviolet light. The species used as positive controls were: *P. gingivalis* (ATCC 33277); *A. actinomycetemcomitans* (ATCC 29522); *T. forsythia* (ATCC 43037) (Nociti et al. 2001).

All examinations were performed by an experienced, blinded investigator.

CT and micro-CT analysis

Computed tomography (CT) (GE Light Speed 64 VCT, General Electric Co., Fairfield, CT, USA) was performed at baseline and month 3 after ligature removal (Fig. 1), following the clinical and microbial examinations. The imaging parameters were as follows: 120 kVp, 350 mA, 0.5 s rotation time and 0.6 mm collimation. After scanning, three-dimensional reconstruction was performed (GE ADW4.3 CT Workstation, USA), and the height of the bone level around the implants ($BH_{(CT)}$, from bottom of the defect to the implant apex in the CT images) was analysed based on four aspects (buccal, lingual, mesial and distal) of each implant. An average of the four sides was then calculated.

After the second CT examination, the animals were sacrificed under general anaesthesia (overdose of sodium pentobarbital, 200 mg/kg/i.v.), and perfused with a fixative solution (10% buffered formalin) through the carotid arteries. A total of 12 block biopsy samples including the implant and the surrounding tissues, were obtained using a diamond saw. All the samples were fixed in a 10% neutral buffered formalin solution for 1 week. The biopsy samples, including the implant, were then wrapped in cling film to prevent the samples from drying out and were subsequently examined using a micro-CT scanner (SCANCO μCT 80; SCANCO Medical AG, Basserdorf, Switzerland). The following imaging settings were used: tube voltage, 80 kV; tube current, 115 μA; integration time, 200 ms; slice width, 25 μm; slice pitch, 30 μm; matrix size, 512 × 512. After scanning, the peri-implant bone architectures were reconstructed using OsiriX software (Atlanta, GA, USA); changes in the post-treatment bone levels of each implant were measured.

Histomorphometric examination

All block biopsies including the implants, were processed for ground sectioning according to the methods described by Donath & Breuner (1982) and in accordance with the protocol by Albouy et al. (2012). For each implant block, four central sections (buccal-lingual plane) were obtained and reduced to a thickness of approximately 20 μm and stained in 1% toluidine-blue solution. Using this technique, old bone stains light blue, whereas newly formed bone stains dark blue (Schenk et al. 1984). The histomorphometric evaluation was performed using a Leica DMRBE microscope (Leica, Heidelberg, Germany) equipped with an imaging system (Q-500 MC; Leica). Four buccal-lingual sections per implant were examined and the heights of bone level ($BH_{(HM)}$, from first bone-to-contact to implant apex) and linear re-osseointegration height ($RH_{(HM)}$, from first BIC to bottom of defect) were measured from the buccal and the lingual sides of each implant, expressed as an average of the two sides.

Statistical analysis

The SPSS 16.0 software (SPSS Inc, Chicago, IL, USA) was used. Mean values for each implant were

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

obtained in each animal. Differences within groups over different time points were evaluated by Friedman test and Wilcoxon signed-rank test, and the Wilcoxon signed-rank test was used to test the differences between groups. Differences were considered statistically significant at $p < 0.05$.

Results

All procedures were well tolerated by the animals, and post-treatment healing was uneventful in all dogs throughout the study.

Histomorphometric findings

Primary outcome: Buccal-lingual sections representing the implants of the two groups are shown in Fig. 3. The degree of osseointegration was evaluated by measuring the data of linear $BH_{(HM)}$ and $RH_{(HM)}$. At month 3, a significant difference of $BH_{(HM)}$ was observed in the plasma group (7.52 ± 0.47 mm) compared with the control group (6.38 ± 0.55 mm) ($p < 0.05$), while the bone level around the implants was not significantly different at baseline (Table 1). The $RH_{(HM)}$ in the plasma group became significantly higher compared with the control group at month 3 (1.52 ± 0.46 *versus* 0.81 ± 0.37, $p < 0.05$). Thus, the higher $BH_{(HM)}$ and $RH_{(HM)}$ demonstrated better bone healing at 3 months post-treatment at the implant site of the plasma group. Higher $BH_{(HM)}$ and $RH_{(HM)}$ were observed in the plasma group (Fig. 3a,b) than that in the control group (Fig. 3c,d).

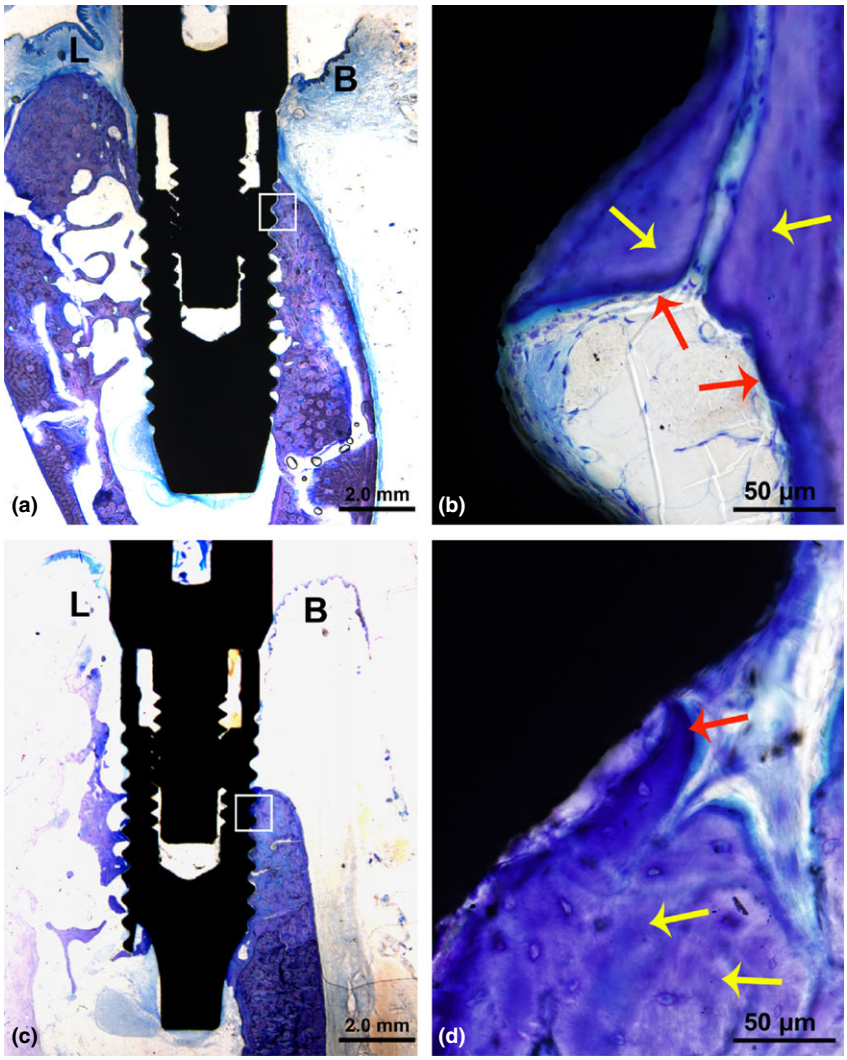

Fig. 3. Histomorphometric sections (bucco-lingual) of the implant with surrounding tissues at month 3 (1% toluidine-blue staining). (a) Sections of the plasma group. Original magnification 1×. (b) Details of (a). Original magnification 40×. (c) Sections of the control group. Original magnification 1×. (d) Details of (c). Original magnification 40×. (a, c) show the heights of bone level ($BH_{(HM)}$) in the two groups. Newly formed bone (dark stained area, red arrows) and old bone (light stained area, yellow arrows) are showed in (b, d). Landmarks for the histomorphometric analysis: B, buccal side; L, lingual side; Scale bars = 2.0 mm (a, c) and 50 μm (b, d).

Clinical findings

The SBI and PD values were significantly lower after treatment in both groups ($P < 0.05$) (Table 2). At baseline, there was no significant difference between the two groups, but data in the plasma group were significantly lower ($p < 0.05$) than the net of the control group at month 3.

Microbial analysis findings

The detection rate of the *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia* results are shown in Table 3. At baseline, the detection rates for the three bacteria were not significantly different between the plasma and the control groups. At month 1, the detection rates for all three species declined significantly in both groups ($p < 0.05$), with the only exception of the detection rates of *A. actinomycetemcomitans* in the control group ($p > 0.05$). At month 3, the detection rates of *P. gingivalis* and *T. forsythia* in the plasma group were significantly lower ($p < 0.05$) than that at baseline, while the significantly declining trend of *P. gingivalis* and *T. forsythia* in the control group stopped at month 2. The detection rates of *P. gingivalis* and *T. forsythia* at months 1, 2 and 3 were significantly higher ($p < 0.05$) in the control group than that of the plasma group, but there was only a significant difference observed regarding the detection of *A. actinomycetemcomitans* between the two groups at month 1 ($p < 0.05$).

Imaging examination findings

CT and micro-CT 3D images are shown in Fig. 4. At baseline, there was no significant differences in the bone height from CT examination ($BH_{(CT)}$) between the plasma group and the control group (5.89 ± 0.85

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Table 1. The bone level in beagle dogs with peri-implantitis treated by non-equilibrium plasma at baseline and month 3

Time	$BH_{(CT)}$ (mm)		$BH_{(HM)}$ (mm)		$RH_{(HM)}$ (mm)	
	Plasma	Control	Plasma	Control	Plasma	Control
Baseline	5.89 ± 0.85	5.62 ± 0.70	–	–	–	–
Month 3	7.22 ± 0.75*,#	6.13 ± 0.69	7.52 ± 0.47#	6.38 ± 0.55	1.52 ± 0.46#	0.81 ± 0.37

Data are presented as mean ± standard deviation.
*$p < 0.05$ *versus* baseline in each group.
#$p < 0.05$ *versus* control at the same points.
$BH_{(CT)}$, Bone height of CT analysis; $BH_{(HM)}$, Bone height of histomorphometric analysis; $RH_{(HM)}$, Height of linear re-osseointegration.

Table 2. The clinical findings in beagle dogs with peri-implantitis treated by non-equilibrium plasma at baseline and month 3

Time	SBI		PD (mm)	
	Plasma	Control	Plasma	Control
Baseline	4 (3—5)	4 (3—5)	4.77 ± 1.45	4.27 ± 1.25
Month 3	1 (0—2)*,#	1.5 (0—2)*	2.52 ± 0.70*,#	3.29 ± 0.58*

Data are presented as median (range) or mean ± standard deviation.
*$p < 0.05$ *versus* baseline in each group.
#$p < 0.05$ *versus* control at the same points.
SBI, sulcus bleeding index; PD, probing depth.

Table 3. The detection rate of the three bacteria *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia* in beagle dogs with peri-implantitis treated by non-equilibrium plasma

Time	*Pg*		*Aa*		*Tf*	
	Plasma	Control	Plasma	Control	Plasma	Control
Baseline	0.67	0.67	0.33	0.33	0.83	0.83
Month 1	0.17*,#	0.33*	0*,#	0.17	0.17*,#	0.50*
Month 2	0.17*,#	0.33*	0.17	0.33	0.33*,#	0.50*
Month 3	0.33*,#	0.50	0.21	0.33	0.33*,#	0.67

*$p < 0.05$ *versus* baseline in each group.
#$p < 0.05$ *versus* control at the same points.
Pg = *P. gingivalis*, *Aa* = *A. actinomycetemcomitans* and *Tf* = *T. forsythia*.

versus 5.62 ± 0.70; Table 1). At month 3, the data of $BH_{(CT)}$ in the plasma group were significantly higher than that in the control group (7.22 ± 0.75 mm *versus* 6.13 ± 0.69 mm; Table 1). This result was consistent with the PD results. The different bone levels of the implants in micro-CT images between the two groups at month 3 can be seen in Fig. 4e–h.

Discussion

A feasible treatment of peri-implantitis must be capable of cleaning contaminated implant surfaces and re-establishing surface characteristics that promoted bone regeneration (Claffey et al. 2008). Currently, new developments regarding the use of non-equilibrium plasmas for the removal of oral biofilms in titanium surfaces and wounds suggest that they may be a visible option in the treatment of peri-implantitis (Isbary et al. 2010, Vogelsang et al. 2010, Koban et al. 2011, Canullo et al. 2013). Thus, the present study is aimed at evaluating the effects of non-equilibrium plasma in the treatment of peri-implantitis in a dog model.

The results of the changes of bone healing before and after treatment were reported as primary findings. At baseline, there was no significant difference in the bone height of the implants between the plasma group and the control group according to the CT outcomes. There was a significantly higher $BH_{(HM)}$ in the plasma group compared with the control group at three months post-treatment. Thus, the higher $BH_{(HM)}$ demonstrated better bone healing at the implants of the plasma group. A larger amount of new bone formation in the bone-to-implant contact surface was also observed in the plasma group (Fig. 3b). These in vivo findings were consistent with the results of in vitro experiments, which showed that non-equilibrium plasma treatment may improve cell adhesion (Naciri et al. 2014). Moreover, these results provided further support for some previous conclusions that after non-equilibrium plasma pre-treatment in vitro, titanium implants or abutments may improve bone formation in vivo (Canullo et al. 2013, Giro et al. 2013). Our findings suggest that the conventional therapy along with plasma application may induce bone re-osseointegration in vivo, although the reason for this is not clear. It is possible that this effect is due to the following: (i) An appropriate set of the plasma operating parameters ($0.1\%O_2$/He, 2 L/min, 8 kV, 8 kHz, 1600 ns pulse width, 3 min irradiation time) was used. Plasma effects can be quite selective under different dose and dose rates, which may result in selective damage to pathogenic microbes without damage to the host (Dobrynin et al. 2009): <1 J/cm^2: bacteria sterilized while normal cells survive; 2–6 J/cm^2: cell proliferation and migration, cancer cell apoptosis; >7 J/cm^2: normal cell death; >10 J/cm^2: cell necrosis. Therefore, further research must identify this dose–effect in order to avoid adverse events; (ii) Surface modification. The in vitro studies have demonstrated that non-equilibrium plasma may reduce the contact angle and promote spreading of osteoblasts on different types of surfaces (such as machined, sandblast-etched and a

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

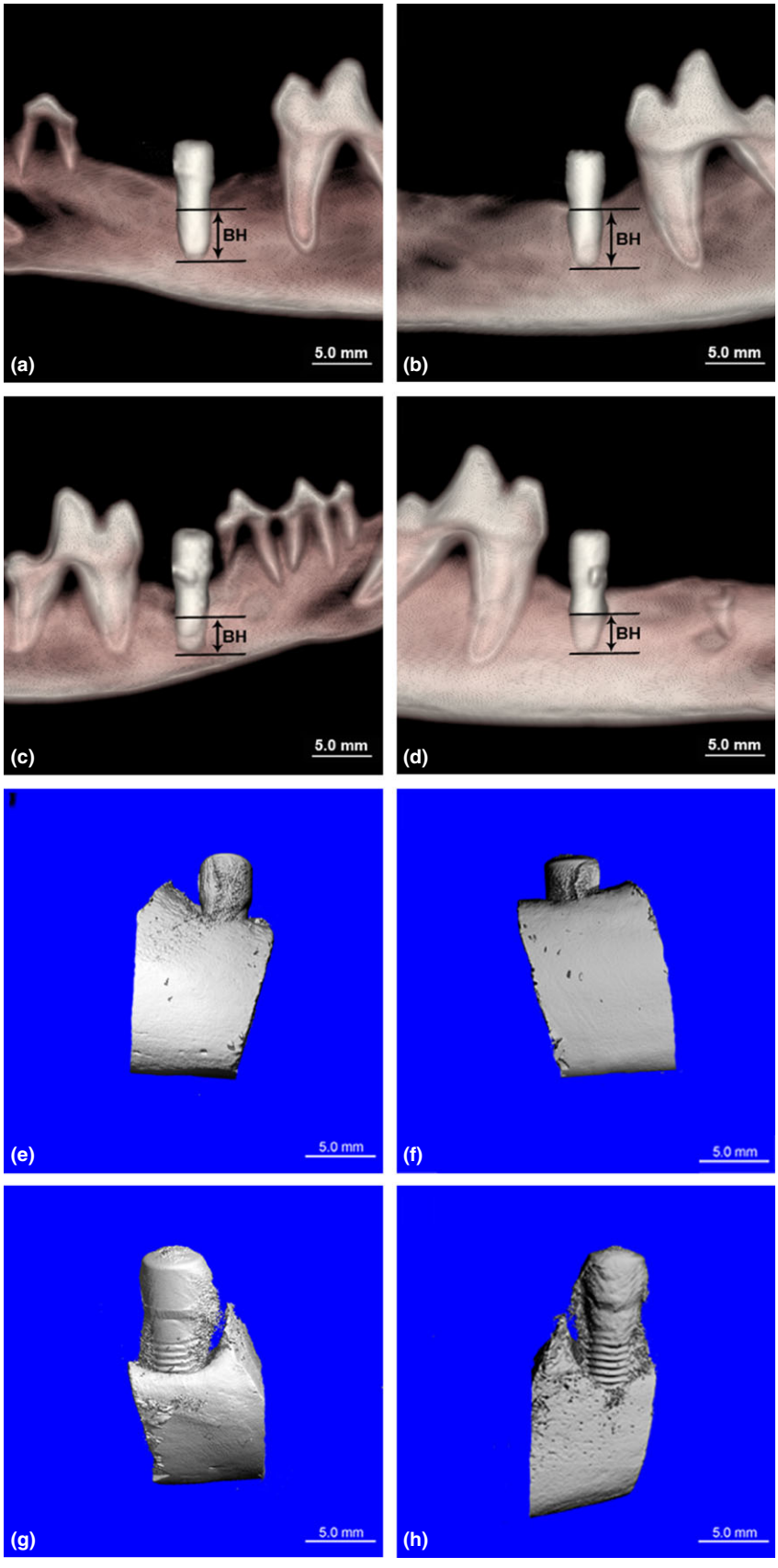

Fig. 4. CT images (a, b, c and d) and micro-CT 3D images (e, f, g and h) of the implant and surrounding tissues. (a) Implant in the plasma group at baseline. (b) Implant in the plasma group at month 3. (c) Implant in the control group at baseline. (d) Implant in the control group at month 3. (e) Buccal aspect of the implant in the plasma group at month 3. (f) Lingual aspect of implant in the plasma group at month 3. (g) Buccal aspect of the implant in the control group at month 3. (h) Lingual aspect of the implant in the control group at month 3. Landmarks: BH, the height of bone around the implant; Scale bars = 5.0 mm.

hydrophilic SLActive® surface) (Duske et al. 2012). In this in vivo study, ANTHOFIT® implants with a BCP® surface consisting of a mixture of hydroxyapatite and beta-tricalcium phosphate, were directly treated in the peri-implantitis dog model using an O_2/He-based non-equilibrium plasma application. The significantly increased height of the bone in the plasma group indicated that non-equilibrium plasma may promote the healing of BCP® surface implants. The effect of non-equilibrium plasma to other implant surfaces, including alumina-blasted/acid-etched (AB/AE) (Integra-Ti™; Bicon LLC, Boston, MA, USA) and calcium-phosphate (CaP) (Integra-CP™; Bicon LLC), have also been reported by Coelho et al. (2012) and Giro et al. (2013), respectively. These authors used an Argon-based non-equilibrium plasma application (Kin-Pen™; INP- Greifswald, Greifswald, M-V, GER) and irradiated the implants surface immediately prior to implant placement in a canine model, which resulted in an improved bone formation. Although the above reports pose good results, the underlying mechanisms regarding the reaction of bone-to-implant following plasma treatment remained unclear. Further in vitro and in vivo studies need to be conducted to explain this interesting phenomenon.

To evaluate the effects of non-equilibrium plasma in the inactivation of microorganisms in the peri-implantitis dog model, we specially designed a new compact non-equilibrium plasma application for this study. We found that the plasma

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

generated is around room temperature and contained numerous short-lived reactive species (excited OH, N_2, N_2+, He and O species). The reactive oxygen species, including excited OH, O and O_3 atoms and metastable state O_2, had millisecond-range lifetimes, which led to key roles in inactivating microorganisms in previous studies (Lu et al. 2008). As plasma is an ionized gaseous medium, it can spread to the surrounding environment rather than travel in straight line, and it can enter the curved, narrow, irregular pores and cracks more easily. Du et al. (2012) found that non-equilibrium plasma can even reach into narrow dentinal tubules during killing *Enterococcus faecalis* in root canals. Hence, non-equilibrium plasma, unlike conventional treatments, might not be largely influenced by bone defect configurations and implant surface characteristics when used in the treatment of peri-implantitis. In addition to the plasma, the conventional therapy included surgical intervention, manual scaling and antibiotic irrigation (0.2% chlorhexidine), which are techniques known to improve access to the peri-implant pocket, improve the clinical symptoms, and inhibit microbial growth (Mombelli 2002, Lindhe & Meyle 2008, Thierbach & Eger 2013).

Porphyromonas gingivalis, *Actinobacillus actinomycetemcomitans* and *Tannerella forsythia* are important pathogeneses that are associated with peri-implantitis (Becker et al. 1990, Nociti et al. 2001, Mombelli & Décaillet 2011). As expected, a better antimicrobial efficacy to these three bacteria was shown in the plasma group at months 1, 2 and 3 (Table 3). The in vivo results seemed to be analogous to the in vitro studies, which that showed the antimicrobial efficacy of non-thermal plasma against dental biofilms on titanium discs (Koban et al. 2011, Rupf et al. 2011). Moreover, the microbial analysis results demonstrate that the non-equilibrium plasma combined with conventional therapy had a more comprehensive and long-lasting effect of sterilization. Thus, non-equilibrium plasma with conventional therapy may be more effective to promote biofilm removal compared to conventional therapy only. We noticed that the subsequent increase in the detection rates occurred in both groups which indicate that recolonization of bacteria occurred on the implant surfaces and reminds us of the regular dental check-ups with or without re-treatments should be considered in the clinical practice.

At baseline, there were no significant differences between the two groups in clinical examines (SBI, PD) and the height of bone level (BH) (Table 1). At month 3, the plasma group showed a significant improvement on clinical exam (SBI, PD) ($p < 0.05$) and a significantly higher BH compared to the control group. These findings indicate that both treatments improved the clinical symptoms; however, the use of non-equilibrium plasma in combination with conventional therapy was more effective.

There are some limitations to this study. One of them was that the treatments started right after the removal of ligatures, without another period for plaque accumulation to continue. Another limitation was the standard exposure time of 3 min, using a plasma application (even though effective), might not be optimal. Furthermore, the small amounts of experimental animals ($n = 6$) especially a low number of implants installed in each dog ($n = 2$) was not appropriate, according to the 3Rs principles. The low numbers of bacterial species detected ($n = 3$) could not entirely represent a complex microbiota of peri-implantitis. In addition, *P. gingivalis* and *A. actinomycetemcomitans* may only be found at a low level of in peri-implantitis lesions (Koyanagi et al. 2010). A limitation of CT was that the spatial resolution was not very precise (0.6 mm). Nonetheless, the results provided sufficient for the preliminary evidence in the potential use of non-equilibrium plasma for peri-implantitis treatment.

Future investigations will focus on the various non-equilibrium plasma treatment parameters in order to determine the optimal conditions. These will include increasing the number of bacteria detected, increasing number of installed implants in each dog, prolonging the observation period, and conducting a more accurate analysis (i.e. the DNA checkerboard, or pyrosequencing techniques for microbiological analysis; the use of cone beam CT instead of CT). Finally, it is necessary to further explain the mechanisms underlying the antimicrobial effect of non-equilibrium plasma on the plasma-implant and plasma-cell interactions.

Conclusion

Within the limits of this study, non-equilibrium plasma treatment as an adjunct to the conventional therapy is a feasible approach for peri-implantitis. Thus, non-equilibrium plasma may enable new routes for the therapy of peri-implant lesions in the future.

Acknowledgements

The authors thank Yubin Xian, Yunhao Qiu and Xing Cao (State Key Laboratory of Advanced Electromagnetic Engineering and Technology, Huazhong University of Science and Technology) for their excellent technical assistance.

References

Albouy, J.-P., Abrahamsson, I. & Berglundh, T. (2012) Spontaneous progression of experimental peri-implantitis at implants with different surface characteristics. An experimental study in dogs. *Journal of Clinical Periodontology* **39**, 182–187.

Becker, W., Becker, B. E., Newman, M. G. & Nyman, S. (1990) Clinical and microbiologic findings that may contribute to dental implant failure. *International Journal of Oral and Maxillofacial Surgery* **5**, 31–38.

Berglundh, T. & Stavropoulos, A. (2012) Preclinical in vivo research in implant dentistry. Consensus of the eighth European workshop on periodontology. *Journal of Clinical Periodontology*, **39**(s12), 1–5.

Canullo, L., Peñarrocha, D., Clementini, M., Iannello, G. & Micarelli, C. (2013) Impact of plasma of argon cleaning treatment on implant abutments in patients with a history of periodontal disease and thin biotype: radiographic results at 24-month follow-up of a RCT. *Clinical oral implants research* **00**, 1–7.

Cao, Y. G., Yang, P., Lu, X. P., Xiong, Z. L., YE, T., Xiong, Q. & Sun, Z. Y. (2011) Efficacy of atmospheric pressure plasma as an antibacterial agent against *Enterococcus faecalis* in vitro. *Plasma Science and Technology* **13**, 93–98.

Claffey, N., Clarke, E., Polyzois, I. & Renvert, S. (2008) Surgical treatment of peri-implantitis. *Journal of Clinical Periodontology* **35**, 316–332.

Coelho, P. G., Giro, G., Teixeira, H. S., Marin, C., Witek, L., Thompson, V. P., Tovar, N. & Silva, N. R. (2012) Argon-based atmospheric pressure plasma enhances early bone response to rough titanium surfaces. *Journal of*

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Biomedical Materials Research Part A **100A**, 1901–1906.

Dard, M. (2012) Methods and interpretation of preclinical performance studies for dental implants. In: Boutrand, J. P. (ed.). *Biocompatibility and Performance of Medical Devices*, pp. 308–344, Cambridge: Woodhead Publishing Ltd.

Dobrynin, D., Fridman, G., Friedman, G. & Fridman, A. (2009) Physical and biological mechanisms of direct plasma interaction with living tissue. *New Journal of Physics* **11**, 115020.

Donath, K. & Breuner, G. (1982) A method for the study of undecalcified bones and teeth with attached soft tissues. The Sage-Schliff (sawing and grinding) technique. *Journal of Oral Pathology* **11**, 318–326.

Du, T. F., Ma, J. Z., Yang, P., Xiong, Z. L., Lu, X. P. & Cao, Y. G. (2012) Evaluation of antibacterial effects by atmospheric pressure non-equilibrium plasmas against *Enterococcus faecalis* biofilms in vitro. *Journal of Endodontics* **38**, 545–549.

Du, T. F., Shi, Q., Shen, Y., Cao, Y. G., Ma, J. Z., Lu, X. P., Xiong, Z. L. & Haapasalo, M. (2013) Effect of modified nonequilibrium plasma with chlorhexidine digluconate against endodontic biofilms in vitro. *Journal of Endodontics* **39**, 1438–1443.

Duske, K., Koban, I., Kindel, E., Schröder, K., Nebe, B., Holtfreter, B., Jablonowski, L., Weltmann, K. D. & Kocher, T. (2012) Atmospheric plasma enhances wettability and cell spreading on dental implant metals. *Journal of Clinical Periodontology* **39**, 400–407.

Esposito, M., Grusovin, M. G. & Worthington, H. V. (2012) Interventions for replacing missing teeth: treatment of peri-implantitis. *Cochrane Database of Systematic Reviews* **1**, Art. No.: CD004970.

Giro, G., Tovar, N., Witek, L., Marin, C., Silva, N. R. F., Bonfante, E. A. & Coelho, P. G. (2013) Osseointegration assessment of chairside argon-based nonthermal plasma-treated Ca-P coated dental implants. *Journal of Biomedical Materials Research Part A* **101A**, 98–103.

Halfmann, H., Bibinov, N., Wunderlich, J. & Awakowicz, P. (2007) A double inductively coupled plasma for sterilization of medical devices. *Journal of Physics D: Applied Physics* **40**, 4145.

Heinlin, J., Isbary, G., Stolz, W., Zeman, F., Landthaler, M., Morfill, G., Shimizu, T., Zimmermann, J. L. & Karrer, S. (2013) A randomized two-sided placebo-controlled study on the efficacy and safety of atmospheric non-thermal argon plasma for pruritus. *Journal of the European Academy of Dermatology and Venereology* **27**, 324–331.

Heitz-Mayfield, L. J. (2008) Peri-implant diseases: diagnosis and risk indicators. *Journal of Clinical Periodontology* **35**(Suppl. 8), 292–304.

Idlibi, A. N., Al-Marrawi, F., Hannig, M., Lehmann, A., Rueppell, A., Schindler, A., Jentsch, H. & Rupf, S. (2013) Destruction of oral biofilms formed in situ on machined titanium (Ti) surfaces by cold atmospheric plasma. *Biofouling* **29**, 369–379.

Isbary, G., Morfill, G., Schmidt, H. U., Georgi, M., Ramrath, K., Heinlin, J., Karrer, S., Landthaler, M., Shimizu, T., Steffes, B., Bunk, W., Monetti, R., Zimmermann, J. L., Pompl, R. & Stolz, W. (2010) A first prospective randomized controlled trial to decrease bacterial load using cold atmospheric argon on chronic wounds in patients. *British Journal of Dermatology* **163**, 78–82.

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. & Altman, D. G. (2011) Animal research: reporting in vivo experiments–the ARRIVE guidelines. National Centre for the Replacement, Refinement and Reduction of Amimals in Research. *Journal of Cerebral Blood Flow & Metabolism* **31**, 991–993.

Kim, G. C., Lee, H. W., Byun, J. H., Chung, J., Jeon, Y. C. & Lee, J. K. (2013) Dental applications of low-temperature nonthermal plasmas. *Plasma Processes and Polymers* **10**, 199–206.

Kim, J. Y., Ballato, J., Foy, P., Hawkins, T., Wei, Y. Z., Li, J. H. & Kim, S. O. (2011) Apoptosis of lung carcinoma cells induced by a flexible optical fiber-based cold microplasma. *Biosensors and Bioelectronics* **28**, 333–338.

Klinge, B., Hultin, M. & Berglundh, T. (2005) Peri-implantitis. *Dental clinics of North America* **49**, 661–676, vii–viii.

Koban, I., Holtfreter, B., Hübner, N. O., Matthes, R., Sietmann, R., Kindel, E., Weltmann, K. D., Welk, A., Kramer, A. & Kocher, T. (2011) Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro -proof of principle experiment. *Journal of Clinical Periodontology* **38**, 956–965.

Koban, I., Jablonowski, L., Kramer, A., Weltmann, K. D. & Kocher, T. (2012) Medical plasma in dentistry: A future therapy for peri-implantitis. In Machala, Z., Hensel, K. & Akishev, Y. (eds.). *Plasma for Bio-Decontamination, Medicine and Food Security*. pp. 190–200. Netherlands: Springer.

Koyanagi, T., Sakamoto, M., Takeuchi, Y., Ohkuma, M. & Izumi, Y. (2010) Analysis of microbiota associated with peri-implantitis using 16S rRNA gene clone library. *Journal of Oral Microbiology* **2**, 1–7. doi: 10.3402/jom.v2i0.5104.

Lang, N. P. & Berglundh, T. (2011) Periimplant diseases: where are we now?-Consensus of the Seventh European Workshop on Periodontology. *Journal of Clinical Periodontology* **38**(Suppl. 11), 178–181.

Laroussi, M. (1995) Sterilization of tools and infectious waste by plasmas. *Bulletin of the American Physical Society* **40**, 1685–1686.

Lindhe, J., Berglundh, T., Ericsson, I., Liljenberg, B. & Marinello, C. (1992) Experimental breakdown of periimplant and periodontal tissues. A study in the beagle dog. *Clinical Oral Implants Research* **3**, 9–16.

Lindhe, J. & Meyle, J. (2008) Peri-implant diseases: consensus report of the sixth European workshop on periodontology. *Journal of Clinical Periodontology* **35**, 282–285.

Lu, X. P., Cao, Y. G., Yang, P., Xiong, Q., Xiong, Z. L., Xian, Y. B & Pan, Y. (2009) An RC plasma device for sterilization of root canal of teeth. *IEEE Transactions on Plasma Science* **37**, 668–673.

Lu, X. P., Ye, T., Cao, Y. G. & Sun, Z. (2008) The roles of the various plasma agents in the inactivation of bacteria. *Journal of Applied Physics* **10**, 053309.

Mazza, J. E., Newman, M. G. & Sims, T. N. (1981) Clinical and antimicrobial effect of stannous fluoride on periodontitis. *Journal of Periodontology* **8**, 203–212.

Mombelli, A. (2002) Microbiology and antimicrobial therapy of peri-implantitis. *Periodontology* **2000**, 177–189.

Mombelli, A. & Décaillet, F. (2011) The characteristics of biofilms in peri-implant disease. *Journal of Clinical Periodontology* **38**(Suppl. 11), 203–213.

Mombelli, A. & Lang, N. P. (1998) The diagnosis and treatment of peri-implantitis. *Periodontology* **2000**, 63–76.

Naciri, M., Dowling, D. & Al-Rubeai, M. (2014) Differential sensitivity of mammalian cell lines to non-thermal atmospheric plasma. *Plasma Processes and Polymers*, **11**, 391–400.

Nociti, F. H., Cesco de Toledo, R., Machado, M. A., Stefani, C. M., Line, S. R. & Gonçalves, R. B. (2001) Clinical and microbiological evaluation of ligature-induced peri-implantitis and periodontitis in dogs. *Clinical Oral Implants Research* **12**, 295–300.

Rupf, S., Idlibi, A. N., Marrawi, F. A., Hannig, M., Schubert, A., Von Mueller, L., Spitzer, W., Holtmann, H., Lehmann, A., Rueppel, A. & Schindler, A. (2011) Removing biofilms from microstructured titanium *ex vivo*: a novel approach using atmospheric plasma technology. *PLoS ONE* **6**, e25893.

Schaudinn, C., Jaramillo, D., Freire, M. O., Sedghizadeh, P. P., Nguyen, A., Webster, P., Costerton, J. W. & Jiang, C. (2013) Evaluation of a nonthermal plasma needle to eliminate *ex vivo* biofilms in root canals of extracted human teeth. *International endodontic journal* **46**, 930–937.

Schenk, R. K., Olah, A. J. & Herrmann, W. (1984) Preparation of calcified tissues for light microscopy. In: Dickson, G. R. (ed.) *Methods of Calcified Tissue Preparation*, pp. 1–56. Amsterdam: Elsevier.

Sladek, R. E. J., Filoche, S. K., Sissons, C. H. & Stoffels, E. (2007) Treatment of Str*eptococcus mutans* biofilms with a nonthermal atmospheric plasma. *Letters in Applied Microbiology* **45**, 318–323.

Sladek, R. E. J., Stoffels, E., Walraven, R., Tielbeek, P. J. A. & Koolhoven, R. A. (2004) Plasma treatment of dental cavities: a feasibility study. *IEEE Transactions on Plasma Science* **32**, 1540–1543.

Slots, J., Ashimoto, A., Flynn, M. J., Li, G. & Chen, C. (1995) Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clinical Infectious Diseases* **20**(Suppl. 2), S304–S307.

Thierbach, R. & Eger, T. (2013) Clinical outcome of a nonsurgical and surgical treatment protocol in different types of peri-implantitis: a case series. *Quintessence international* **44**, 137–148.

Vogelsang, A., Ohl, A., Steffen, H., Foest, R., Schroeder, K. & Weltmann, K.-D. (2010) Locally resolved analysis of polymer surface functionalization by an atmospheric pressure argon microplasma jet with air entrainment. *Plasma Processes and Polymers* **7**, 16–24.

de Waal, Y., van Winkelhoff, A. J., Meijer, H. J., Raghoebar, G. M. & Winkel, E. G. (2012) Differences in peri-implant conditions between fully and partially edentulous subjects: a systematic review. *Journal of Clinical Periodontology* **40**, 266–286.

Address:
Yingguang Cao
Department of Stomatology
Tongji Hospital
Tongji Medical College
Huazhong University of Science and Technology
1095 Jiefang Avenue, Wuhan
430030, China
E-mail: cyg0729@tjh.tjmu.edu.cn

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Clinical Relevance

Scientific rationale for the study: So far, there is not reliable evidence to show which conventional treatment is the most effective management of peri-implantitis. Due to new results regarding the use of nonequilibrium plasmas in the removal of oral biofilms on titanium surfaces and wounds, there is evidence suggesting that nonequilibrium plasmas may be a viable option in the treatment of peri-implantitis. This study aims to evaluate the effects of nonequilibrium plasma in the treatment of peri-implantitis in beagle dogs.

Principal findings: Nonequilibrium plasma as an adjunct to conventional therapy showed a significant improvement of bone re-osseointegration, and reduced the detection of subgingival bacteria (*P. gingivalis* and *T. forsythia*). In addition, clinical examination (the sulcus bleeding index, probing depth) outcomes were significantly improved when compared to conventional therapy alone. No significant difference was observed between the two methods with respect to the detection of *A. actinomycetemcomitan* at 3 months post-treatment ($p > 0.05$).

Practical implications: Nonequilibrium plasma may be a feasible approach in the treatment of peri-implantitis and may enable new routes for the therapy of peri-implant lesions.

© 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd