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Surface conditioning with cold argon plasma and its effect on the osseointegration of dental implants in miniature pigs

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ABSTRACT

Purpose: Successful implant therapy is based on fast, safe, and predictable osseointegration. Several surface modifications have been introduced to improve the bone-to-implant interaction. This *in vivo* study evaluates the impact of plasma surface conditioning on early wound healing and osseointegration.
Materials and methods: A total of 16 dental implants with a sand-blasted and acid-etched surface were conditioned with cold atmospheric plasma prior to insertion in the frontal bone of four miniature pigs. Sequential fluorescence labeling was administered to label bone metabolism, and after 8 weeks, bone blocks were harvested for radiological, histological, and histomorphometrical evaluation.
Results: The plasma conditioning had no impact on the morphology of the implant surface. The bone-to-implant contact ratio was 90.4% and 86.5%, the interthread bone density 72.5% and 63.4%, and the periimplant bone density 60.5% and 61.1%, in the plasma conditioned group and control group, respectively. Concentric bands of fluorescence enrichment indicated a chronological and homogenous mineralization of newly formed bone. No unwanted periimplant side effects were detected.
Conclusion: The increased parameters for osseointegration in this in vivo study merit further investigation in prospective clinical trials.

1. Introduction

Successful implant therapy is based on fast, safe, and predictable osseointegration. The surface properties of dental implants cause an immediate host reaction and thus affect the subsequent signal transduction cascades that should lead to osseointegration (Coelho et al., 2009). Therefore, to induce a favorable early host-to-implant response, several modifications were investigated to enhance the geometrical and physico-chemical properties of titanium implant surfaces in order to achieve higher primary stability and faster and more reliable osseointegration (Albrektsson and Wennerberg, 2004a,b; Abrahamsson and Berglundh, 2009). As a result of this research, it is now generally accepted that implant surfaces must be moderately roughened and that physico-chemical modifications can further optimize healing (Coelho et al., 2009; Dohan Ehrenfest et al., 2010). The finding that surface modification in terms of surface energy and wettability play an important role in osseointegration is now generally accepted (Mendonca et al., 2008; Junker et al., 2009; Gittens et al., 2014; Ricci and Alexander, 2016). Therefore, it can be assumed that current implant surfaces still offer the potential for improvement. Reasons for that assumption are that, on one hand, our knowledge of the respective surface-bound host reaction is limited, and, on the other hand, the production of complex multi-scaled surfaces with reproducible physico-chemical properties is a technical challenge (Rupp et al., 2018). Additionally, combinations of texture and chemistry are proprietary product–specific processes controlled by manufacturers and are not general-purpose processes widely available to the surgical community. Therefore, it is of great clinical interest to investigate simple and readily available chairside concepts that allow for surface modification of any implant surface (Coelho et al., 2012). In particular, the use of plasma for biomedical applications shows beneficial effects involving changes in both energy and the

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chemistry of surfaces due to high concentrations of reactive species that are generated and thus have a positive effect on early wound healing and implant-to-bone interaction (Coelho et al., 2012; Duske et al., 2012; Laroussi et al., 2012; Danna et al., 2015).

Plasma is described as a partially ionized gas with ions, electrons, and uncharged particles (atoms, molecules, and radicals). Plasma can be differentiated into two types: thermal, and non-thermal (**c**old) **a**tmospheric **p**lasma (CAP), with a temperature of less than 40 °C at the point of application. Several *in vitro* studies proved that the wettability and cell spreading on titanium surfaces is enhanced by plasma modification (Duske et al., 2012). It was revealed that plasma treatment leads to increased cell proliferation, cell spreading, and synthesis of proteins of the extracellular matrix, and that the osteoblast-like cells form more developed networks (Kim et al., 2015; Lee et al., 2015, 2017). Another study points out that osteoblasts show higher cell adhesion and positive cell morphology outcomes on plasma conditioned titanium surfaces compared to untreated surfaces (Canullo et al., 2017a,b).

However, *in vivo* investigations concerning the effect of CAP for conditioning of dental implants prior to insertion are rare at present. The aim of this study was to compare the difference in osseointegration of untreated as well as plasma-modified titanium dental implants in an animal model using miniature pigs. These findings may improve the success of clinical applications of dental implant rehabilitation due to faster and more predictable osseointegration.

2. Materials and methods

2.1. Implants and protocol for plasma surface conditioning

In this study, titanium dental implants (3.8 mm diameter, 9 mm length) with an abrasive-blasted, acid-etched Promote® plus surface (CAMLOG® SCREW-LINE, Camlog Biotechnologies AG, Basel, Switzerland) were used. The protocol for the CAP-surface conditioning was applied chairside immediately prior to insertion of the implants. Therefore, each implant in the test group was treated for 240 s, evenly distributed from the neck to the tip, 60 s per quadrant, with a distance of 7 mm using an atmospheric pressure plasma jet (kINPen Med, Neoplas Tools GmbH, Greifswald, Germany). The plasma flame was generated by a driving voltage of 115–230 V, a driving frequency of 20 kHz combined with an argon gas pressure of 2 bar and a flow rate of 4.3–4.4 L/min (Fig. 1). The argon gas had a purity of >99.998%.

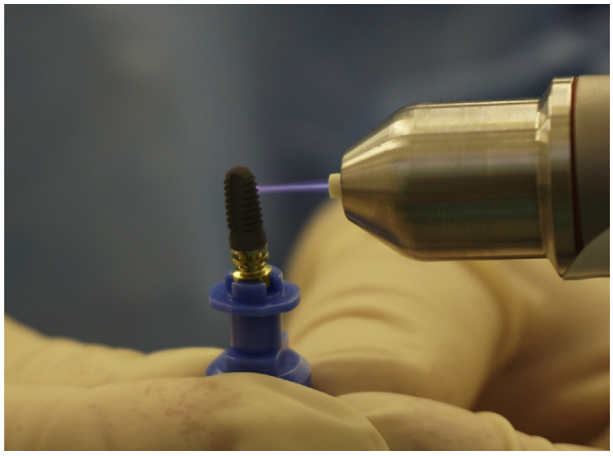

Fig. 1. Surface conditioning of the implants immediately prior to insertion with cold plasma. Standardized plasma application was done with even distribution for 60 s per quadrant with a distance of 7 mm.

2.2. Scanning electron microscopy

To investigate the surface morphology with and without CAP modification, five implants per group were investigated *in vitro* by scanning electron microscopy (SEM) (XL30CP, Philips Electron Optics GmbH, Kassel, Germany). The surface conditioning prior to SEM was performed according to the protocol mentioned above. Sputtering was not necessary. The scans were performed with a voltage of 10–15 kV using magnifications from 50 to 10,000.

2.3. Experimental animals and surgical procedure

The animal experiments were conducted according to the European Animal Welfare Legislation and in accordance with the European Communities Council Directive (2010/63/EU). The study protocol was approved by the Directorate of Food-Chain and Animal Healthcare, Hungary (Approval No. PEI/001/961-2/2013) and was conducted in compliance with the appropriate ARRIVE guidelines. The study included 4 female miniature pigs with mature skeletons (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) with an average age of 10 months and an average weight of 22–24 kg. Miniature pigs are most suitable for this study due to their bone metabolism characteristics, which are similar to those of humans. The animals were kept under conventional conditions, equipped with straw in groups of four, with food and water *ad libitum*.

A single surgeon with an experienced team performed all operations on the animals in a standardized manner. Animals were sedated via intramuscular injection of 10 mg/kg ketamine and 1.5 mg/kg midazolam. Anesthesia was maintained with ketamine intravenously. The miniature pigs were placed in a prone position and prepared for an aseptic operation on the frontal bone. Additionally, 6 ml of articaine 4% was injected for local anesthesia at the surgical site. A median incision 8 cm in length was made at the forehead, and the calvaria was approached subperiosteally. Above the suture frontoparietalis, the bone lateral to the frontal sinus was prepared for implant insertion according to the manufacturer's instructions. In every animal, two CAP-conditioned implants and two control implants were placed bilaterally. The insertion was performed with a torque ratchet so that the implant surface was completely covered and only the machined implant shoulder extended to the bony surface. The closure screws were inserted and a two-layer skin closure was performed. Analgesia was induced by intramuscular injection of 500 mg metamizole twice a day for 5 days, and a prophylactic antibiotic (125 mg/d ceftriaxone 125 mg for 5 days) was given. In order to label bone metabolism, Alizarin Red was injected 30 mg/kg intraperitoneally after 2, 4, and 6 weeks. After 8 weeks, euthanasia was performed and bone blocks of $1.5 \times 1.5 \times 2$ cm with the implants in the center were harvested and stored in formalin 4%.

2.4. Radiographic and histologic analysis

The fixed specimens were dehydrated using ascending concentrations of alcohol in an embedding station (Pool of Scientific Instruments, Type 1.42.00, PSI Grünewald, Laudenbach, Germany) and embedded in methyl-methacrylate (Sigma–Aldrich, St. Louis, MO, USA). Periapical radiographs were performed using 2×3-cm x-ray films, a tube voltage of 65 kV, and a current of 7 mA (Gendex Expert® DC, Kavo Dental GmbH, Biberach, Germany) in order to evaluate the periimplant bone tissue. The probes were cut parallel to the longitudinal axis of the implants with a diamond-coated saw (Cut-grinder primus Diamant, Walter Messner, Oststeinbek, Germany), and four 40-µm-thick non-decalcified specimens per implant were prepared (DP-U4, Struers GmbH, Willich, Germany).

First, an evaluation by fluorescence microscopy was performed (Axio Oberserver.Z1 and Axio Cam and Axio Vision, Carl Zeiss Mikroskopie, Jena, Germany). Subsequently, Toluidine blue staining and light microscopy were performed. For histomorphometric analysis, images were assessed digitally using Leica QWin Standard (V 3.2.0 Leica Microsystems Imaging Solution, Cambridge, UK). The bone-to-implant contact ratio (BIC) was defined as the length of bone-to-implant contact in relation to the implant perimeter starting at the shoulder of the implant. The interthread bone density (ITBD) was defined as the area of bone inside the threads in relation to the interthread area in the five most central threads on both sides. The periimplant bone density (PIBD) was defined as the bone area in relation to the total area surrounding the implants up to a lateral distance of 1 mm, again at the five most central threads on both sides. The analyses were conducted by the same researcher, who was blinded as to which group each sample belonged.

2.5. Statistical analysis

All data were collected and presented as the mean and the standard error of the mean. The data sets were tested for normal distribution and homogeneous variance. Data sets were analyzed using two-way analysis of variance (ANOVA) followed by the Tukey HSD *post hoc* test (SPSS Statistics 20.0, IBM Corporation, Armonk, NY, USA). The significance level was set to a p value of 0.05.

3. Results

3.1. Surface morphology

The application of CAP did not lead to any macroscopically recognizable changes in the implant surfaces. Scanning electron microscopy was performed with both CAP-conditioned implants as well as non-conditioned ones. At varying magnifications, no differences between the two surfaces could be detected. Even at high magnifications, the microporosity of the Promote® plus surface appeared unchanged compared to the control (Fig. 2). The insertion of the implants, which was performed by a torque ratchet, had not been influenced by the CAP conditioning. The primary stability had not been affected by application of the plasma.

3.2. Baseline data

No wound healing disturbance or any other adverse side effects occurred. Every implant of the test group as well as of the control group showed sufficient primary stability. The success rate of implant placement was 100%. The data for every animal and every implant could be included in the results.

3.3. Osseointegration

The results of the histomorphometric measurements of osseointegration are presented in Table 1. The CAP-conditioned implants showed an increased BIC (90.4% ± 1.24%) compared to the control group (86.5% ± 1.23%, $p = 0.053$). This result indicated a trend towards improved osseointegration, but the level of significance was $p = 0.53$, which is a slightly nonsignificant difference. The interthread bone density was significantly higher ($p = 0.002$) with the CAP-conditioned implants (72.47% ± 5.21%) than with the control implants (63.36% ± 6.21%). Fig. 3 shows histological sections of the bone-to-implant interface stained in Toluidine blue, demonstrating newly formed bone at the implant surface and between the threads (Fig. 3A and D). The newly formed bone appears darker than mature bone. The bone tissue contained a high number of osteoblasts, comparable to the adjacent mature bone. Fluorescence microscopy showed an enrichment of red fluorescence at the implant surface, indicating bone growth at 2, 4, and 6 weeks after insertion. In some areas, the fluorescence appeared in three bands of equal distances, which suggests chronologic and uniform bone growth within this time period. In some areas, only two bands of fluorescence dye appeared (Fig. 3B and C). Furthermore, in some areas, the bands were not as clearly circumscribed, which means that bone growth was more unsteady in these areas between weeks 2 and 6. However, no relevant discrepancy between the controls and the CAP-conditioned implants appeared. In fact, a discrepancy of fluorescence enrichment was noticeable at different areas of the implants, but these findings did not have any distinct or determinate discrepancy of distribution.

3.4. Periimplant bone tissue

Radiologically, the cortical layer differs from the cancelous part of the bone. The periimplant cancelous tissue appeared homogeneous and presented a density comparable to that of the surrounding tissue in both treatment groups. Furthermore, the tissue looked homogenous at every section of the implants. There were no radiological signs of periimplant resorption or osteolysis (Fig. 4). Histologically, with all implants the periimplant cancelous bone was denser and more compact than the spongy bone tissue that was not in close proximity to the implant (Fig. 4). Comparing the two treatment groups, the PIBD was comparable (60.48 ± 5.03% vs. 61.14 ± 6.10%) (Table 1). At the tip of some implants, independent of the treatment group, the bone tissue appeared less dense, and the trabeculae were thinner (Fig. 4). No histological signs of acute or chronic inflammation, osteolysis, or resorption occurred in any specimen.

4. Discussion

The aim of the present study was to evaluate the effect of surface CAP treatment with regards to osseointegration of titanium implants in a miniature-pig model. In our study, osseointegration of all implants was successful. After 8 weeks, the titanium implant surfaces with additional CAP-treatment demonstrated increased BIC and ITDB compared to that in the control group.

With regard to the test set-up chosen for this study, the miniature pig animal model is common and well-validated with regard to bone morphology, anatomy, healing, and remodeling, and therefore it is very well suited to modeling the condition of human bones and the healing process of dental implants (Pearce et al., 2007; Fabbro et al., 2017). To the best of our knowledge, our study is the first to collect data on plasma conditioning in the miniature pig model. The data collected here therefore form the basis for making the miniature pig model comparable for the investigation of plasma conditioning in the future. Different bone-to-implant parameters as indicators for osseointegration have been investigated. The definition of the term “osseointegration” was clarified by Albrektsson and Branemark et al. (Albrektsson et al., 1981) as the direct contact between a loaded implant surface and bone at a microscopic level. Therefore, high BIC values and interthread bone density are generally considered prerequisites for implant stability in order to allow functional dental reconstruction. These parameters indicate the osteogenic potential of implant surfaces (Bernhardt et al., 2012).

In order to provide a meaningful baseline for the measured BIC values, it is necessary to assess values that can be determined for comparable implant surfaces without plasma treatment. Comparable data for BIC at different titanium implant surfaces (blasted wrinkled, HA-coated, double acid etched, anodic sparked deposition, SLA) were presented by Fabbro et al., with values between 76%

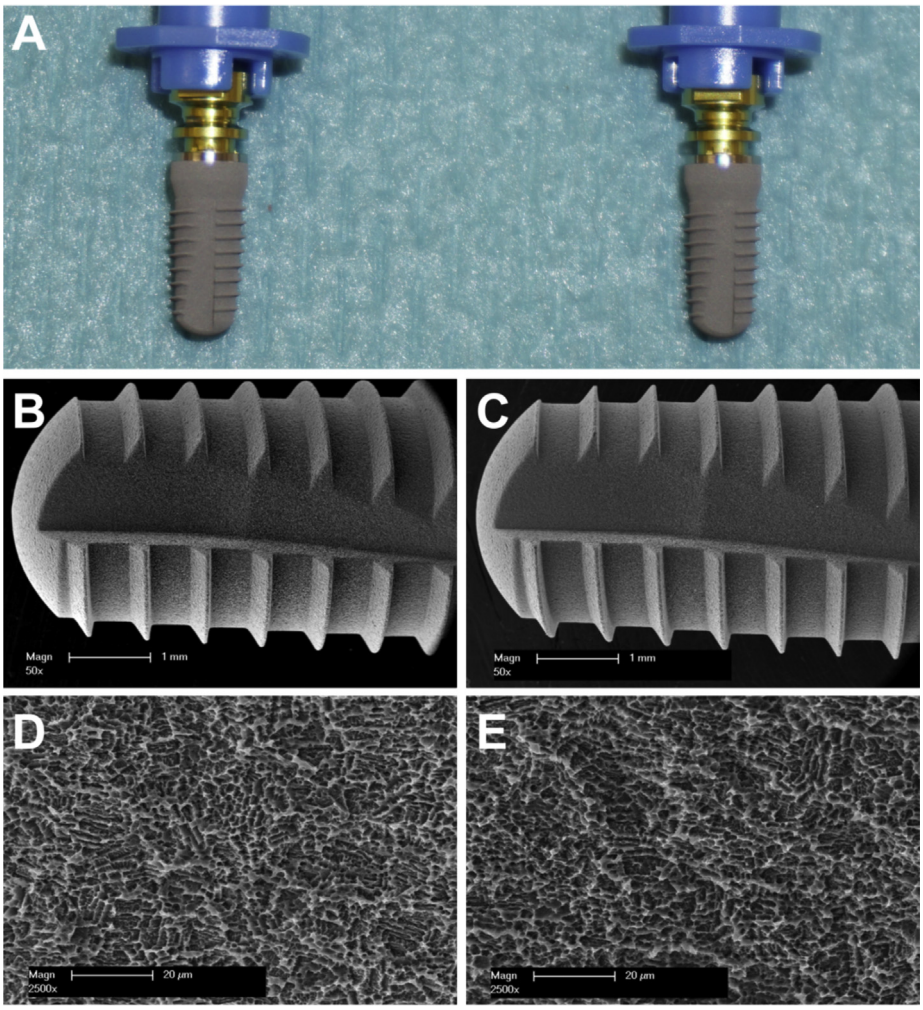

Fig. 2. Plasma conditioning does not affect the macroscopic and microscopic morphology of the surface of the implants. The upper row shows a non-conditioned (left) and conditioned implant (right) immediately after application of the plasma (A). The middle and lower row show scanning electronic microscopy of untreated (B+D) and plasma-modified surfaces (C+E). n = 5 implants per treatment group.

Table 1
Histomorphometric parameters for osseointegration at plasma-conditioned implants and control implants.

Histomorphometric parameters	Plasma-conditioned implants (n = 8)	Control implants (n = 8)	Significance level (p)
Bone-to-implant contact ratio (BIC), %	90.42 ± 1.24	86.48 ± 1.23	0.053
Interthread bone density (ITBD), %	72.47 ± 5.21	63.36 ± 6.21	**0.002**
Periimplant bone density (PIBD), %	60.48 ± 5.03	61.14 ± 6.10	0.819

A significance level $p \leq 0.05$ was considered to be statistically significant and is indicated in boldface type.

und 83%, with no significant differences between the tested surfaces (Fabbro et al., 2017) but a value for SLA close to our findings. Another similar BIC for untreated SLA surfaces was described by Cochran et al. (BIC 68% after 4 weeks of submerged healing and 79% after 8 weeks of immediate loading) in the mandible (Cochran et al., 2016). Therefore, it can be assumed that the BIC values obtained for untreated SLA-implant surfaces might be in accordance with the presently known data. However, even restricting the comparable results to the minipig model results in difficulties with regard to different locations of the implant site (mandible or maxilla), which are not equal when comparing the aforementioned studies.

These findings are in accordance with other studies describing the effect of CAP treatment in animal models. A similar protocol for CAP conditioning was used in a study in beagle dogs that investigated the surface properties and osseointegration of titanium implants of different diameters. In that study, plasma conditioning resulted in a modification of the ion spectrum of the surface and a higher bone-to-implant-contact ratio after 3 weeks, but not after 1 week (Coelho et al., 2012). The same group investigated osseointegration of calcium-phosphate–coated dental implants, with and without plasma conditioning of the implant surface, according to the previously described protocol. As in the first study mentioned, they stated that there was no difference in the BIC ratio after 1 week, However, a significantly higher BIC ratio was found in the plasma modified implant group after 3 weeks (Giro et al., 2013). Teixeira at al. evaluated implants that were treated with non-thermal plasma in comparison to an untreated control in beagle dogs. The non-thermal plasma in this study was

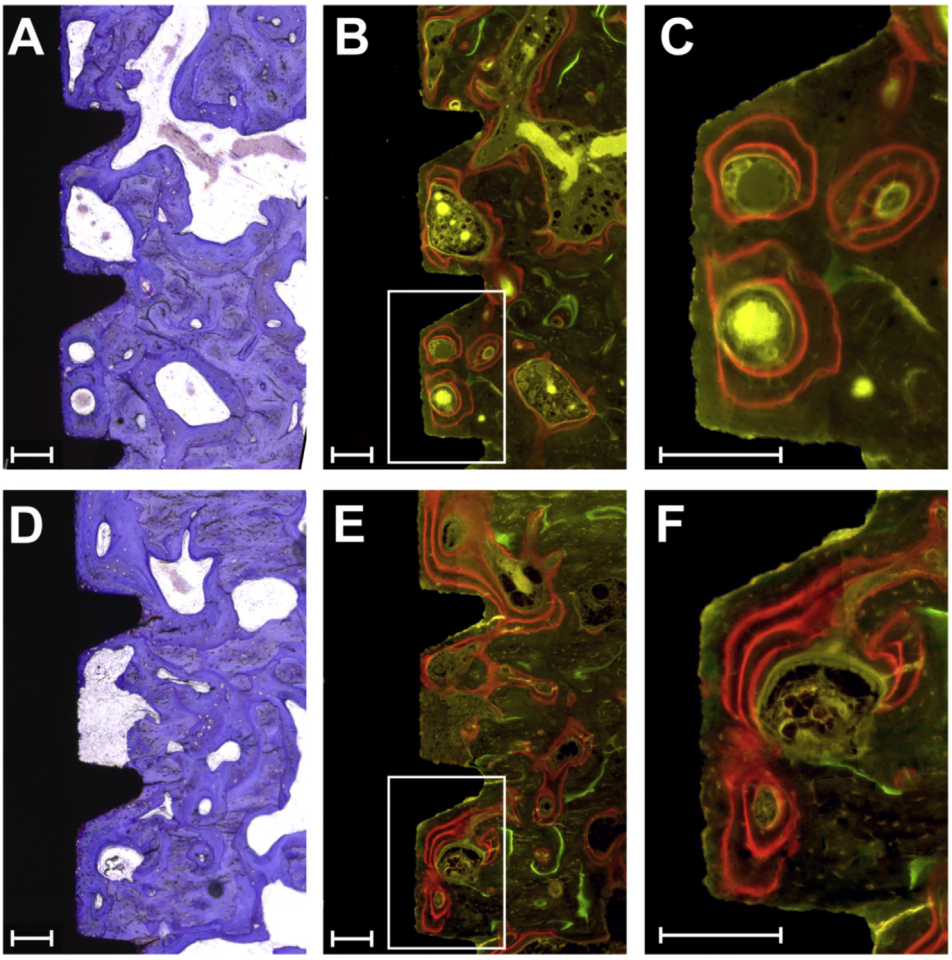

Fig. 3. Histological evaluation of the bone-to-implant interface with Toluidine blue staining and fluorescence microscopy. Newly formed bone is labeled by the enrichment of red fluorescence. The implant in the upper row is untreated (A–C), while the lower row shows a plasma modified implant (D–F). The scale bar is 200 μm.

applied for 20 or 60 s per quadrant, respectively. Both plasma groups showed substantially higher surface energy levels, lower amounts of adsorbed C-species, and significantly higher removal torque levels after 2 and 4 weeks in comparison to the control group (Teixeira et al., 2012). Another investigation was done with untreated and argon-based plasma conditioned implants in the tibiae of dogs. Plasma application was completed either 30 days earlier or immediately prior to the investigation, and the implants had been stored in their original vials until surgery. The results showed that the surface's elemental chemistry was modified by the plasma and lasted for 30 days after treatment, resulting in improved biomechanical fixation and higher bone formation 2 weeks after implantation when compared to the control group (Guastaldi et al., 2013). This applied not only to titanium surfaces that had been treated with atmospheric plasma, but also to dental implants made of zirconia with different surface roughness values. In a study using the tibiae of rabbits, the plasma treatment on zirconia implants made the surface more hydrophilic and enhanced the osseointegration of the implants without changing the micro-topography (Shon et al., 2014).

As the micro- and macro-morphology of the surface remains unchanged by the CAP conditioning, its impact on the physico-chemical surface energy can be postulated. Therefore, CAP treatment might have an effect, especially in the early stages of the osseointegration process, leading to a higher BIC ratio in general. Another hypothesis for the clinical use of cold atmospheric plasma is that argon plasma can be used for disinfection of contaminated titanium surfaces as it appears under periimplantitis conditions, and that the treatment improves the subsequent bony regeneration due to its beneficial surface characteristics (Canullo et al., 2017a,b). Investigations for that hypothesis were completed with titanium discs with different surfaces that had been contaminated with bacteria frequently found in periimplantitis. Plasma treatment was performed in an argon plasma chamber for 12 min. The following microbiological evaluation proved that argon plasma led to an effective reduction of bacterial contamination (Annunziata et al., 2016). In contrast to this study, which used an atmospheric pressure plasma jet, the discs were treated in a plasma chamber for a longer duration, which might have caused a stronger effect from the plasma. The advantage of a pen is that it can be used chairside, and therefore for implants which stay *in vivo*, as the pen can be used intraorally.

Another *in vitro* study with titanium surfaces of different roughness values and different hydrophobia revealed that roughness has a greater impact on bacterial adhesion than surface treatment with argon plasma. In that study, the surface was exposed to argon plasma discharge on all sides for 4 min on three occasions (Amoroso et al., 2006). A similar conclusion was made in a study using contaminated titanium discs, which were cleaned by air polishing, CAP, or a combination of both methods. The plasma treatment is less effective for decontamination and does not lead to better subsequent spreading of osteoblast-like cells (Matthes et al.,

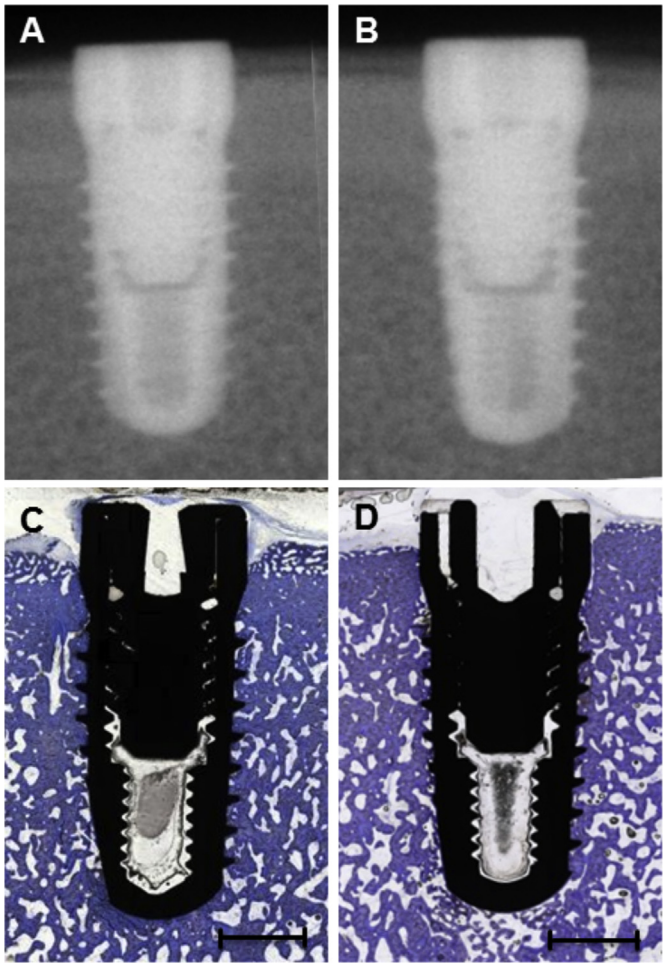

Fig. 4. Overview of the implants and adjacent bone tissue by periapical radiograph (A and B) and Toluidine blue staining (C and D). Plasma modification (in the right column) of the surface does not influence the structure or quantity of the adjacent tissues. The scale bar is 2 mm.

2017). The hypothesis that decontaminated implant surfaces provide a higher capacity for re-osseointegration, if they have been treated with cold plasma, has not yet been investigated *in vivo*.

In this study, the postoperative observation time was 8 weeks, which is a typical period for submerged healing in two staged implant procedures in bones of good quality. After 8 weeks, the BIC was 86.5% in the control group and 90.4% in the plasma-modified test group. We had applied red fluorescence staining after 2, 4, and 6 weeks after implant placement to gain information about the chronological sequence of bone formation at the bone–implant interface. Our results showed three bands of red fluorescence at the implant threads in both treatment groups without finding a relevant discrepancy. In fact, a discrepancy of fluorescence enrichment was noticeable at different surface areas of the implants, without a distinct and determinate distribution. Studies that performed histological investigations at early time points concluded that the BIC ratio is higher after 2, 3, and 4 weeks (Coelho et al., 2012; Teixeira et al., 2012; Giro et al., 2013; Guastaldi et al., 2013). It remains unclear whether this seems to be an indication for earlier implant loading in two staged procedures. In fact, immediate or early loading of implants depends on primary stability, which is influenced mainly by the bone density and implant geometry.

Repeated *in vivo* radiographs provide another possible method for the longitudinal evaluation of periimplant bone formation in individuals. However, three-dimensional techniques such as cone beam computed tomography cause periimplant artifacts and are not appropriate for periimplant diagnostics. Histological evaluation therefore remains the gold standard for evaluation of osseointegration. Sequential fluorescence staining is helpful for evaluating time-dependent bone formation, but definite histological investigation requires histological preparations after varying observation times with a larger number of experimental animals.

5. Conclusion

Plasma conditioning does not lead to any remarkable changes in macroscopic and microscopic surface morphology. However, CAP conditioning prior to insertion does result in a higher bone-to-implant contact ratio and interthread bone density. Fluorescence labeling does not suggest that CAP conditioning leads to significantly faster or stronger bone adherence and mineralization. The slightly higher osseointegration parameters might indicate a beneficial effect that is worth further investigation within the scope of prospective clinical trials.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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