Aus der Poliklinik für Zahnärztliche Chirurgie und Aufnahme
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Peri-implantitis treatment and prevention: the role of implant material and surface modifications.

Habilitationsschrift

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vorgelegt von
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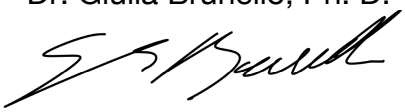
SWORN STATEMENT

I hereby declare in lieu of an oath that I have written this Habilitation thesis without any unauthorised assistance. Furthermore, I affirm in lieu of an oath that the literature used has been mentioned in full. I affirm in lieu of an oath that ethical principles as well as the recommendations for ensuring good scientific practice have been observed and that this Habilitation thesis has not yet been submitted to any other faculty. Finally, I affirm that there are no other Habilitation procedures initiated or unsuccessfully terminated.

Düsseldorf, 7th March 2023

Dr. Giulia Brunello, Ph. D.



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SUMMARY

Titanium dental implants represent a valid treatment option for replacing missing teeth. However, plaque-induced peri-implantitis constitutes a common complication, that could lead to implant failure. Non-surgical therapies have been demonstrated to elicit limited effects and a surgical access is frequently required for its treatment. A crucial step is represented by the implant surface decontamination. Various protocols have been proposed, including mechanical debridement and the use of antiseptics. However, there is no consensus on which is the best approach and each method presents potential shortcomings.

Rotary burs could affect the integrity of dental implants when used for implantoplasty during the surgical treatment of peri-implantitis. Therefore, in this cumulative Habilitation thesis, sonic diamond tips were tested and found to be more conservative in terms of structure loss.

Furthermore, chlorhexidine (CHX) has been widely used in the prevention, treatment and maintenance phase of peri-implant diseases. However, its usage has been associated with several side effects. To overcome these drawbacks, low-concentration CHX mouthwashes have been introduced. CHX at low concentration in combination with cetylpyridinium chloride as adjunctive proved antimicrobial activity *in vitro* and exhibited reduced cytotoxic effect on both fibroblasts and osteoblast-like cells compared to CHX at higher concentration.

Zirconia implants are emerging as promising alternatives to conventional titanium implants. A two-year prospective study was previously conducted by our group. Considering the lack of long-term data, a retrospective 9-year follow-up study was performed and data included in the present Habilitation thesis. An overall stability of the results between 2 and 9 years of follow-up was observed, with only one implant failure in this time lapse and no additional case of peri-implantitis, despite numerous mechanical and technical complications.

ZUSAMMENFASSUNG

Titan-Implantate stellen eine valide Behandlungsoption für den Ersatz fehlender Zähne dar. Plaque-induzierte Periimplantitis ist jedoch eine häufige Komplikation, die zum Verlust des Implantats führen kann. Nicht-chirurgische Therapien haben nachweislich nur eine begrenzte Wirkung, und zur Behandlung ist häufig ein chirurgischer Zugang erforderlich. Ein entscheidender Schritt ist die Dekontamination der Implantatoberfläche. Es wurden verschiedene Protokolle vorgeschlagen, darunter mechanische Reinigung und die Verwendung von Antiseptika. Es besteht jedoch kein Konsens darüber, welche Methode die beste ist, und jede Methode weist potenzielle Mängel auf.

Rotierende Bohrer könnten die Unversehrtheit von Implantaten beeinträchtigen, wenn sie bei der chirurgischen Behandlung von Periimplantitis für Implantatplastik verwendet werden. Daher wurden in dieser kumulativen Habilitationsarbeit Schalldiamantspitzen getestet, die sich in Bezug auf den Strukturverlust als konservativer erwiesen. Darüber hinaus wurde Chlorhexidin (CHX) in der Vorbeugung, Behandlung und Erhaltungsphase von periimplantären Erkrankungen häufig eingesetzt. Die Verwendung von CHX wurde jedoch mit verschiedenen Nebenwirkungen in Verbindung gebracht. Um diese Nachteile zu überwinden, wurden niedrig konzentrierte CHX-Mundspülungen eingeführt. CHX in niedriger Konzentration in Kombination mit Cetylpyridiniumchlorid als Zusatzstoff erwies sich *in vitro* als antimikrobiell wirksam und zeigte im Vergleich zu CHX in höherer Konzentration eine geringere zytotoxische Wirkung sowohl auf Fibroblasten als auch auf osteoblastenartige Zellen.

Zirkoniumdioxid-Implantate entwickeln sich zu einer vielversprechenden Alternative zu herkömmlichen Titan-Implantaten. Eine zweijährige prospektive Studie wurde zuvor von unserer Gruppe durchgeführt. In Anbetracht des Mangels an Langzeitdaten wurde eine retrospektive 9-Jahres-Follow-up-Studie durchgeführt, deren Daten in die vorliegende Habilitationsschrift einfließen. Es wurde eine allgemeine Stabilität der Ergebnisse zwischen zwei und neun Jahren Nachuntersuchung beobachtet, mit nur einem Implantatverlust in diesem Zeitraum und keinem weiteren Fall von Periimplantitis, trotz zahlreicher mechanischer und technischer Komplikationen.

LIST OF THE SUMMARIZED WORKS

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2. Kathrin Becker, Giulia Brunello, Luisa Scotti, Dieter Drescher, Gordon John. Efficacy of 0.05% chlorhexidine and 0.05% cetylpyridinium chloride mouthwash to eliminate living bacteria on in situ collected biofilms: An in vitro study. Antibiotics (Basel). 2021;10(6):730. DOI: 10.3390/antibiotics10060730
3. Giulia Brunello[§], Kathrin Becker[§], Luisa Scotti, Dieter Drescher, Jürgen Becker, Gordon John. The effects of three chlorhexidine-based mouthwashes on human osteoblast-like SaOS-2 cells. An in vitro study. Int J Mol Sci. 2021;22(18):9986. DOI:10.3390/ijms22189986 ([§] The authors contributed equally)
4. Giulia Brunello[§], Kathrin Becker[§], Luisa Scotti, Dieter Drescher, Jürgen Becker, Gordon John. Effect of three chlorhexidine-based mouthwashes on human gingival fibroblasts: an in vitro study. Appl Sci. 2022;12(5):2417. DOI: 10.3390/app12052417 ([§] The authors contributed equally)
5. Giulia Brunello, Nicole Rauch, Kathrin Becker, Ahmad-Reza Hakimi, Frank Schwarz[§], Jürgen Becker[§]. Two-piece zirconia implants in the posterior mandible and maxilla: a cohort study with a follow-up period of 9 years. Clin Oral Implants Res. 2022;33(12):1233-1244. DOI: 10.1111/clr.14005 ([§] The authors contributed equally)

INTRODUCTION

Titanium dental implants represent a valid treatment choice for replacing missing teeth. The aging of the population and of the related dental disorders, such as edentulism, as well as the growing request for predictable and aesthetic solutions are deemed to be responsible for the expansion of the global dental implant market. In particular, in 2021 the European dental implant market was worth USD 1.4 billion and its size is estimated to reach USD 2.5 billion by 2028, exhibiting a compound annual growth rate (CAGR) of 8.4% (BlueWeave Consulting 2022).

Several strategies have been developed over the years to improve dental implant osseointegration. Both subtractive (e.g. sand blasting, acid etching) and additive methods (e.g. anodization, plasma-spraying) have been applied to modify implant surface, aiming at increasing the roughness, improving the corrosion resistance, altering the surface energy and/or the surface chemical composition (Matos 2021).

Daily oral care at home and professional supportive care are fundamental contributors to oral health, as well as they play a crucial role in the long-term success of dental implant treatments. Indeed, there is evidence that poor plaque control and absence of regular maintenance therapy are associated with higher peri-implantitis susceptibility (Berglundh, Armitage et al. 2018).

Plaque-induced peri-implantitis constitutes one of the most common complications in implant dentistry (Derks and Tomasi 2015). It is characterized by a progressive peri-implant bone loss and could lead to implant failure. Despite positive clinical results have been obtained with moderately rough implant surfaces in terms of osseointegration, these may facilitate the accumulation of plaque when exposed to the oral cavity, thus affecting the progression of peri-implantitis.

In case of peri-implantitis, a surgical approach is frequently needed and can be accompanied by the modification of the morphology of the exposed part of the implant by means of implantoplasty, in order to favour the resolution of the inflammation and to reduce the risk of recurrence (Khoury, Keeve et al. 2019).

Chemical products have also been proposed for the prevention, treatment and maintenance of peri-implant diseases. Among these, CHX-based products are commonly used, despite numerous related side effects have been reported (James,

Worthington et al. 2017). To overcome these drawbacks, new formulations characterized by low-concentration of CHX, alone or with other adjunctive agents, have been introduced. However, data on the antimicrobial efficacy and safety of these products are lacking. Indeed, since the antimicrobial activity is usually associated with the disrupt cell membranes, toxicity for different oral cells could also be a concern.
Zirconia dental implants are emerging as promising alternatives to conventional titanium implants, which have been dominating the market in the last decades. It has been postulated that zirconia implants might perform better, owing to reduced plaque accumulation and subsequent reduced risk of peri-implantitis. However, long-term data are lacking and it is not possible at the moment to conclusively draw conclusions regarding this complex phenomenon (Thiem, Stephan et al. 2022).

Aims of this cumulative Habilitation thesis were:

- to assess *in vitro* a new method for performing implantoplasty on titanium implants (i.e. diamond sonic tips followed by finishing Arkansas burs) and to compare it to conventional implantoplasty using a sequence of tungsten carbide egg-shaped burs and Arkansas burs, in terms of treatment time, weight loss, surface roughness, implant wear, and fracture resistance;
- to test *in vitro* the antibacterial activity of different commercially available mouthwashes containing CHX at different concentrations, alone or in combination with CPC, against *in situ* collected biofilm grown on different substrates, i.e. hydroxyapatite and micro-rough titanium disks, representative of teeth and implants surfaces, respectively;
- to investigate *in vitro* the impact of various commercially available mouthwashes containing CHX at different concentrations, alone or in combination with cetylpyridinium chloride (CPC), on osteoblast-like cells and fibroblasts in terms of cell viability, cytotoxicity and apoptosis;
- to retrospectively evaluate the clinical outcomes after 9 years of follow-up of two-piece zirconia implants inserted in the posterior jaws and restored with full-ceramic single crowns.

STATE OF THE ART

Titanium dental implants

Titanium dental implants and peri-implant diseases

Modern implant dentistry started in the 1960s thanks to the contribution of Professor P.I. Brånemark from the University of Gothenburg, who firstly discovered in rabbit studies that titanium was structurally integrated into living bone. This phenomenon characterized by the direct bone-to-implant contact was called osseointegration, and was demonstrated for the first time in nondecalcified histologic sections by the other pioneer of implant dentistry, Professor A. Schroeder from the University of Bern (Buser, Sennerby et al. 2017).

Since their introduction in the 1960s and 1970s, dental implants and their insertion protocols have undergone a progressive evolution and have completely revolutionised the rehabilitation of fully and partially edentulous patients. They are now considered a highly predictable option for replacing missing teeth, with numerous clinical studies reporting a 10-year survival rate above 90% (Moraschini, Poubel et al. 2015, Buser, Sennerby et al. 2017, Howe, Keys et al. 2019).

However, implant-supported restorations are not free from complications. Considering the increasing in the demand for dental implants, the complication rate may raise in the future. Two main types of complications can be distinguished: biological and mechanical/technical complications. The first are associated with inflammatory/infectious lesions affecting peri-implant tissues, while mechanical/technical complications include implant fracture, screw or abutment fracture, occlusal screw loosening, chipping or fracture of the restoration, as well as loss of retention of the prosthesis (Pjetursson, Asgeirsson et al. 2014, Berglundh, Armitage et al. 2018, Heitz-Mayfield and Salvi 2018). Aesthetic outcomes and complications have also been taken into account in several studies (Pjetursson, Asgeirsson et al. 2014).

Major attention is here dedicated to biological complications, in particular peri-implant mucositis and peri-implantitis. Peri-implant mucositis is described as a plaque-associated reversible inflammatory lesion confined to the peri-implant soft tissues in the

absence of loss of supporting bone or continuing marginal bone loss (Heitz-Mayfield and Salvi 2018). Peri-implantitis, instead, is defined as a pathological condition occurring in peri-implant tissues, characterised by inflammation of the peri-implant mucosa and progressive loss of the supporting bone, that could lead to the failure of the implant (Schwarz, Derks et al. 2018) (Figure 1).

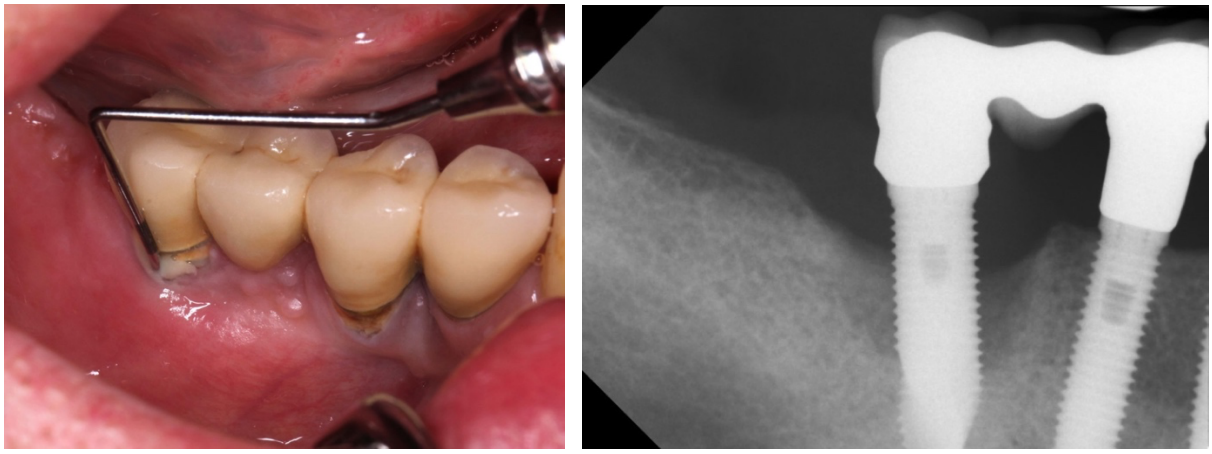

Figure 1: Representative case of peri-implantitis diagnosed 12 years after implant placement, as confirmed by increased probing depth (7 mm), suppuration (left) and radiological evidence of bone loss (right). (Own illustrations)

As regards the prevalence of peri-implant diseases, there is a great variability among the studies, owing to the wide variety of disease definitions and different study population selection (Salvi, Cosgarea et al. 2017). Meta-analysis estimated weighted mean prevalence of peri-implant mucositis of 29.48% (95% Confidence Interval, CI: 22.65–36.32]) and 46.83% (CI: 38.30–55.36) at implant and patient level, respectively. Lower values were reported for peri-implantitis, with weighted mean prevalence of 9.25% (CI: 7.57–10.93) at implant level and 19.83% (CI: 15.38–24.27) at patient level (Lee, Huang et al. 2017).

Bone loss represents the principal parameter to differentiate peri-implant mucositis from peri-implantitis, whose diagnosis requires not only a clinical examination but also a radiologic investigation. Ideally, in order to make a correct diagnosis of peri-implantitis, it is recommended to take baseline radiographs and probing values at the end of the prosthetic rehabilitation. Furthermore, x-rays should also be taken after a certain time from the prosthetic loading in order to have bone level reference following the physiological bone remodelling process (Berglundh, Armitage et al. 2018).

It is assumed that peri-implant mucositis precedes peri-implantitis, hence its early detection and treatment are fundamental to prevent the development of peri-implantitis. However, the clinical and histopathological conditions underlying the evolution from one pathology to the other are not yet fully understood (Berglundh, Armitage et al. 2018).

Plaque accumulation is considered the main etiological factor responsible for both periodontal and peri-implant diseases. Despite they share some similarities in terms of etiology and corono-apical development, these pathologies exhibited different progression patterns (Kotsakis and Olmedo 2021). Indeed, data suggests a faster progression of peri-implantitis than that observed in periodontitis, with a great individual variability in the progression rate (Berglundh, Armitage et al. 2018).

As bacterial colonisation of the peri-implant sulcus is necessary to trigger the pathology, it is considered the primary target their therapy. The pathogenic role of individual bacterial species is unclear, but it is likely that, as in the case of periodontitis, the mere presence of pathogenic species is not sufficient for the onset of the disease. Other elements are deemed to play a role, such as the presence of risk factors and the host predisposition. History of severe periodontitis, inadequate plaque control, and no regular maintenance are considered risk indicators for peri-implantitis, while data on smoking habits and diabetes mellitus are inconclusive (Berglundh, Armitage et al. 2018). The lack of keratinised mucosa around implants may also compromise the long-term stability of peri-implant tissues. Although the role of keratinized mucosa as risk indicator for peri-implantitis remains to be determined, its presence seems to be advantageous in terms of patient comfort during at-home oral hygiene manoeuvres and ease of plaque removal (Berglundh, Armitage et al. 2018). Submucosal cement remnants and implant placement in a position that impedes correct oral hygiene procedures and maintenance may also represent potential risk factors for peri-implantitis (Schwarz, Derks et al. 2018). As regards occlusal overload, its impact on peri-implant bone loss is still controversial (Di Fiore, Montagner et al. 2022). Finally, recent studies have investigated the potential influence of metal particle release on peri-implant bone loss, since these particles are suspected to induce and maintain tissue inflammation. To what extent the release of metal particles and ions in the surrounding tissues can trigger peri-implant inflammation is a current issue of debate (Kotsakis and Olmedo 2021).

Treatment of peri-implantitis

The aim of the treatment of peri-implantitis is to arrest the progressive peri-implant bone loss by controlling the bacterial infection responsible for tissue destruction. An effective treatment should lead to the decrease or resolution of the bleeding on probing (BOP) at the affected sites, as well as to a reduction of the pocket depth (Sanz and Chapple 2012).

Both non-surgical and surgical methods have been proposed, mostly derived or modified from procedures already validated in periodontology (Renvert and Polyzois 2015). Non-surgical approaches represent the first choice for the treatment of peri-implantitis; however, a surgical treatment becomes frequently indicated in case of recurrence of bleeding and suppuration (Khoury, Keeve et al. 2019).

Non-surgical approaches aim to eliminate the bacterial biofilm in a minimally invasive way, without the elevation of a flap, and to intervene on the risk factors, such as improving the patient adherence to oral hygiene regimens or correcting the design of the prostheses to enable optimal cleaning (Renvert, Hirooka et al. 2019). Despite it represents an indispensable preliminary phase and may enable the complete resolution even of advance lesions, such as after the successful removal of gross residues of cements (Wilson, Valderrama et al. 2015, Dalago, Schuldt Filho et al. 2017), in the majority of the cases a subsequent surgical therapy is required.

In the most recent version of the German guidelines for the treatment of peri-implant infections at dental implants (AWMF 2022), the success and the clinical stability of the results (> 6 months) after non-surgical therapy were classified as prognostically unfavourable, especially in case of initial probing depth (PD) values above 7 mm (Schwarz, Sculean et al. 2005, Schwarz, Bieling et al. 2006, Sahm, Becker et al. 2011, John, Sahm et al. 2015). Therefore, a re-evaluation of the treatment success after non-surgical therapy of peri-implantitis was recommended after 6 months at the latest (AWMF 2022).

Independently of the selected surgical approach, due to the biofilm-associated etiology of peri-implantitis, the success of the therapy largely relies in the effective removal of the biofilm from the implant surface (Baima, Citterio et al. 2022). Surface decontamination and conditioning are critical steps to render the implant surface compatible

with tissue healing and possible re-osseointegration. As stated in the report developed by the working group 4 during the World Dental Federation (FDI) consensus meeting in 2019 (Khoury, Keeve et al. 2019), no decontamination protocol resulted to be superior over the others based on the existing evidence, that also failed to show the impact of a particular protocol on surgical therapy. Even though there is no standardized protocol for peri-implantitis treatment, a broad range of surface decontamination methods have been proposed, and can be classified into mechanical, chemical or physical methods (Rakašević and Gabrić 2021). These methods can be used alone or in combination among each other.

The mechanical removal of the granulation tissue, for instance with titanium curettes, as well as the mechanical decontamination of the implant surface are frequently described. Decontamination can be performed mechanically with plastic, carbon or titanium curettes, ultrasonic or sonic scalers, titanium brushes, as well as air-powder abrasive systems (Louropoulou, Slot et al. 2014). Ideally these mechanical methods should be able to effectively remove the biofilm, without altering the characteristics of the implant surface, except when explicitly required, such as in case of implantoplasty (Rakašević and Gabrić 2021).

Surface decontamination can also be performed with chemical methods (Schwarz, Schmucker et al. 2015, Rakašević and Gabrić 2021). Several products have been tested, including ethylenediaminetetraacetic acid (EDTA) gel, citric acid, local antiseptics (e.g. chlorhexidine, hydrogen peroxide, triclosan, taurolidine), and local antibiotics (e.g. metronidazole, minocycline, doxycycline). Alternatively, physical methods such as diode or Er:YAG (erbium-doped: yttrium, aluminium and garnet) laser or photodynamic therapy have also been proposed, which, however, were not found to lead to clinically superior results (Meyle 2012).

The decontamination of the surface is a crucial step in all the surgical strategies, whose common goal is the elimination of peri-implant pocketing and BOP. The surgical approaches can be grouped in three main categories, i.e. access flap, resective approaches (with or without osseous recontouring), or reconstructive procedures (Schwarz, Alcoforado et al. 2021, Karlsson, Trullenque-Eriksson et al. 2022). The selection of the approach is based on the extent and morphology (i.e., supracrestal and/or intrabony defects) of the defect, as well as on the location of the affected implant (Schwarz, Alcoforado et al. 2021).

Access flap consists in the elevation of a flap allowing the decontamination of the implant surfaces, which is then repositioned at the pre-surgical level. This approach should be limited to those cases in which the infra-osseous component of the defect is minimal and soft tissue quality is adequate. Contrary to access flaps, when resective strategies are applied, the flap is apically repositioned at the end of the surgery and sharp edges of the underlying bone can be eliminated to favour flap adaptation (Karlsson, Trullenque-Eriksson et al. 2022). Resective surgery is usually preferable in case of supercrestal defects. The presence of the prosthetic suprastructure may negatively affect the quality of both access flap and resective surgery and, if possible, it is recommended to remove it during the surgery (Khoury, Keeve et al. 2019) (Figure 2).

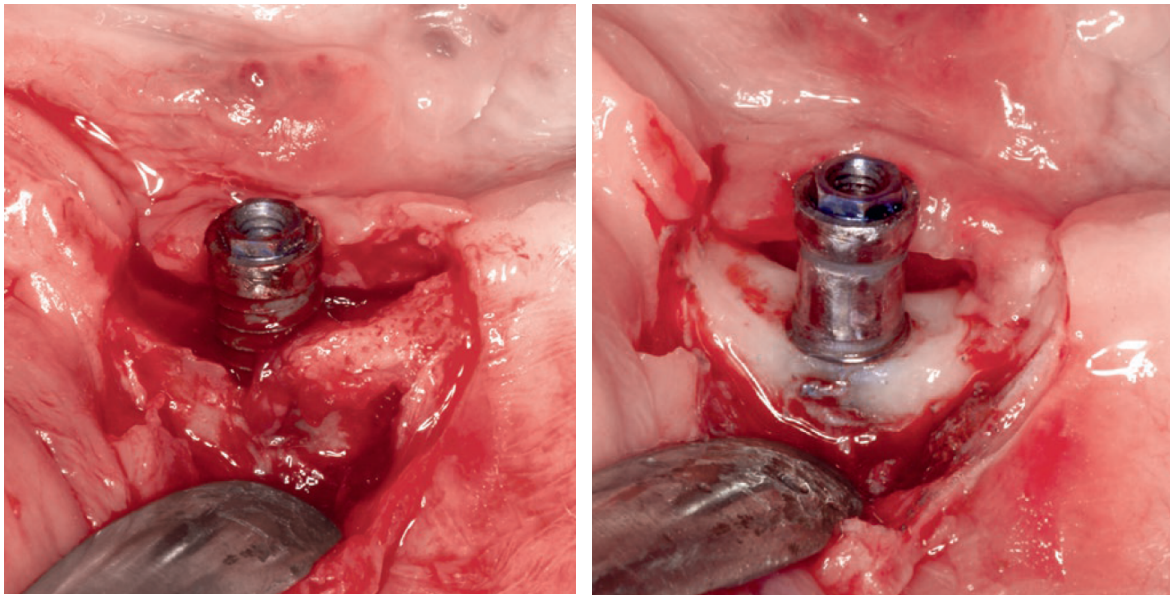

Figure 2: Resective surgical treatment of peri-implantitis (left), characterized by bone recontouring and implantoplasty performed with diamond bur and subsequent Arkansas polishing bur (right). The suprastructure was removed to allow a better access to the implant surface. (Own illustrations)

Regenerative procedures could be indicated to correct peri-implantitis-associated angular bony defects. Reconstructive approaches could require the use of different biomaterials, such as bone substitute materials, barrier membranes, bioactive agents or their combination (Karlsson, Trullenque-Eriksson et al. 2022).

Access flaps and resective approaches were demonstrated to be responsible of a more pronounced mucosal recession as compared with reconstructive procedures (Schwarz, Alcoforado et al. 2021), which might limit their application in aesthetic areas.

The modification of the macro- and micro-roughness of implant surfaces by means of implantoplasty has been proposed in combination with both non-regenerative and regenerative treatments (Ramanauskaite, Daugela et al. 2016, Schwarz, Alcoforado et al. 2021). It consists in the elimination of the exposed implant threads and in smoothing and polishing the resulting surface. It aims to effectively eliminate the biofilm attached to the exposed implant surface and to hamper bacterial re-colonisation of the surface itself, thus reducing the risk of recurrence (Toma, Behets et al. 2018).

When used in combination with resective surgical therapy, it exhibited significant improvement in clinical and radiographic parameters compared to resective therapy alone without implantplasty (Romeo, Ghisolfi et al. 2005, Romeo, Lops et al. 2007). Furthermore, it was demonstrated to be effective also in combination with bone regeneration (Matarasso, Iorio Siciliano et al. 2014), with good clinical outcomes even at a 7-year follow-up (Schwarz, John et al. 2017).

Surgical treatment of peri-implantitis might require the augmentation of non-mobile keratinised mucosa, to facilitate adequate oral hygiene maneuvers, improve aesthetics and/or promote the health and stability of peri-implant soft tissues (Khoury, Keeve et al. 2019). To this aim, various techniques have described, including the combination of coronally advanced flap and connective tissue graft (Figure 3).

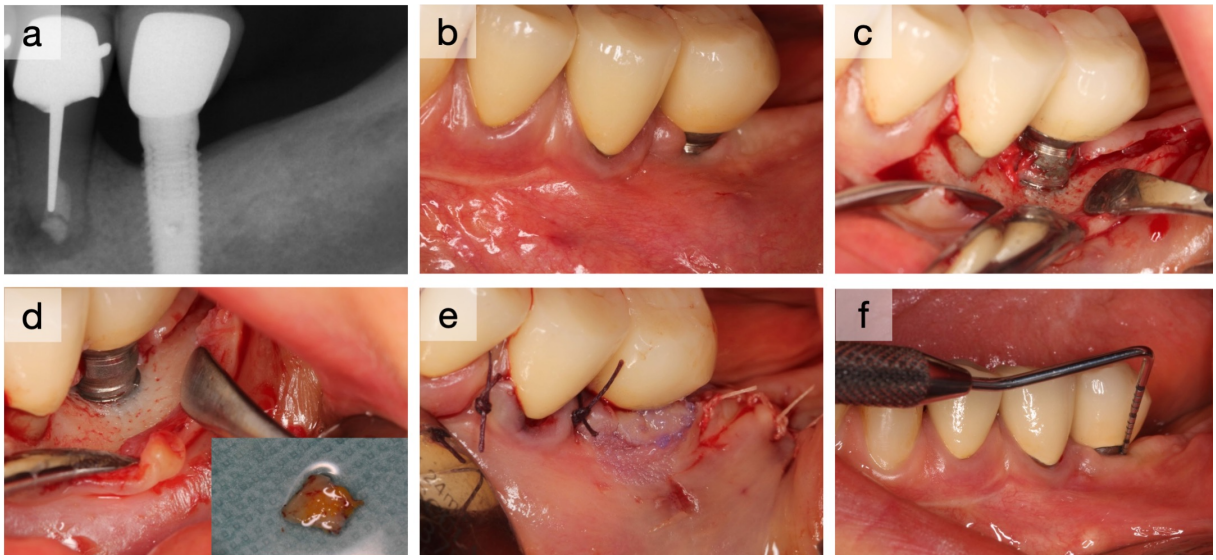

Figure 3: Surgical treatment of peri-implantitis treated with implantoplasty, coronally advanced flap and connective tissue graft: a) preoperative intraoral radiographic image; b) preoperative clinical image; c) flap elevation; d) implantoplasty and harvested connective tissue graft (bottom right image); e) coronally advanced flap; f) clinical image at 9-month follow-up, showing increased keratinized soft tissue width, reduced mucosal recession and PD equal to 3 mm. (Kind courtesy Prof. J. Becker)

Finally, it is worth noting that, to achieve the long-term stability of the results after the treatment of peri-implantitis, the inclusion of the patients in professional hygiene and control maintenance recall programs is fundamental (Roccuzzo, Layton et al. 2018, Khoury, Keeve et al. 2019). However, the compliance with supportive periodontal/peri-implant therapy is generally unsatisfactory (Amerio, Mainas et al. 2020).

Ceramic dental implants

Main features and development

Ceramic implants are becoming an increasingly popular alternative to commonly used moderately-rough titanium dental implants. Their market size was estimated at USD 3,861.34 million in 2022 and it is expected to attain substantial growth in the next few years, reaching USD 14,197.20 million by 2030 with a CAGR of 17.67% (Reportlinker 2023). This trend can be explained by the increasing requests from the patients for metal-free solutions, and by the appealing aesthetics due to their tooth like-colour (Cionca, Hashim et al. 2017, Roehling, Schlegel et al. 2018, Sanz, Noguerol et al. 2019, Kohal and Dennison 2020).

Beside favourable optical properties, zirconia seems to be less prone to bacterial biofilm formation compared with titanium (Roehling, Schlegel et al. 2018). In addition, a stronger mucosal sealing has been observed in presence of zirconia implants (Liñares, Grize et al. 2016, Lee, Ryu et al. 2019). These properties could be particularly beneficial at the trasmucosal portion of the implants, where resistance to bacteria adhesion and colonization could minimize the risk of the onset and progression of peri-implant pathologies. The risk to exacerbate peri-implant inflammation due to the release of titanium wear particles is also avoided with zirconia implants. However, the latter contain other metals, especially zirconium and aluminium, whose release has to be further investigated (Kotsakis and Olmedo 2021).

The first ceramic implants were made of aluminium oxide (Al_2O_3), also known as alumina. Despite they demonstrated good osseointegration, their use was rapidly abandoned due to their poor mechanical properties and related load-induced implant fractures (Roehling, Schlegel et al. 2018).

These first experiences with ceramic implants were not encouraging and led the manufacturers to withdraw them from the market. However, in the 1990s the introduction of a new ceramic material, i.e. zirconium dioxide (ZrO_2), opened new possibilities in implant dentistry. Zirconium dioxide, commonly referred to as zirconia, exhibited higher biomechanical properties as compared to other ceramic materials, allowing it to resist to oral occlusal forces (Roehling, Schlegel et al. 2018).

Nowadays, zirconia represents the material of choice for the production of ceramic implants. Although also other zirconia ceramic compositions have been tested in pre-clinical studies, the materials that have been used in clinical studies are generally yttria-stabilized tetragonal zirconia polycrystal (Y-TZP) and alumina-toughened zirconia (ATZ), which present advanced mechanical properties (Kohal and Dennison 2020).

The rapid evolution in the materials and in the production processes has led one side to the drastic improvement of zirconia implants, on the other side these continuous renewals have a negative impact on the clinical relevance of the investigations (Thiem, Stephan et al. 2022). Indeed, the outcomes reported in the literature are largely based on zirconia implants not available on the market (Pieralli, Kohal et al. 2017, Roehling, Schlegel et al. 2018).

Interestingly, what emerges from a systematic review on 1,128 zirconia implants and 741 patients is a higher implant survival rate in case of commercially available (CA) implants. As compared to CA implants, the non-commercially available (NCA) ones presented a higher percentage of both early (5.8% vs 1.6%) and late (2.6% vs 0.6%) failures (Roehling, Schlegel et al. 2018). Considering the similar surface roughness between the two groups, the better performances of CA implants cannot be justified by quantitative surface roughness. This finding could rather be explained by the higher implant fracture rate registered for NCA implants compared with CA ones (3.4% vs 0.2%).

Despite a single roughness parameter is not sufficient to adequately characterize the complex surface micro-topography, experimental studies have reported comparable osseointegration properties between micro-rough sandblasted and acid-etched zirconia and titanium implants, with mean areal roughness (Sa) of 0.6-0.7 μm and 1.3 μm, respectively (Roehling, Schlegel et al. 2018).

It has to be noted that the first generations of zirconia implants were limited to a one-piece design, mainly due to major concerns related to the mechanical resistance of the material. However, the reduced prosthetic flexibility, the risk for unwanted immediate loading and their limited applicability in some clinical situations, such as in case of simultaneous bone regeneration, have pushed the development of two-piece zirconia implants (Becker, John et al. 2017, Pieralli, Kohal et al. 2017, Roehling, Schlegel et al. 2018) (Figure 4). The introduction of two-piece implants is more recent and, as

a consequence, there is still a lack of information on their long-term clinical outcomes (Thiem, Stephan et al. 2022).

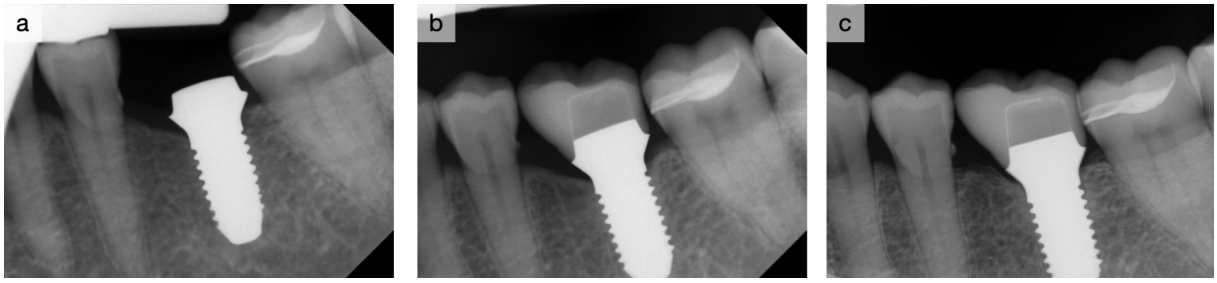

Figure 4: Intraoral radiographs of a one-stage two-piece zirconia implant: a) transmucosal healing; b) 6-month after full-ceramic crown fitting; c) 9-year follow-up. (unpublished images of a patient of the zirconia implant clinical trial, Original work 5, kind courtesy Prof. J. Becker)

When considering CA zirconia implants, a 1-year survival rate of 98.3% (CI: 97.0–99.6) was estimated. Further, meta-regression analysis estimated higher survival rates for Y-TZP vs ATZ and for one-piece vs two-piece zirconia implants, despite these differences were not significant ($p > 0.05$) (Roehling, Schlegel et al. 2018).

Ceramic implants and peri-implant diseases

Peri-implant infections have been reported not only on titanium implants, but also on zirconia implants (AWMF 2022, Thiem, Stephan et al. 2022) (Figure 5).

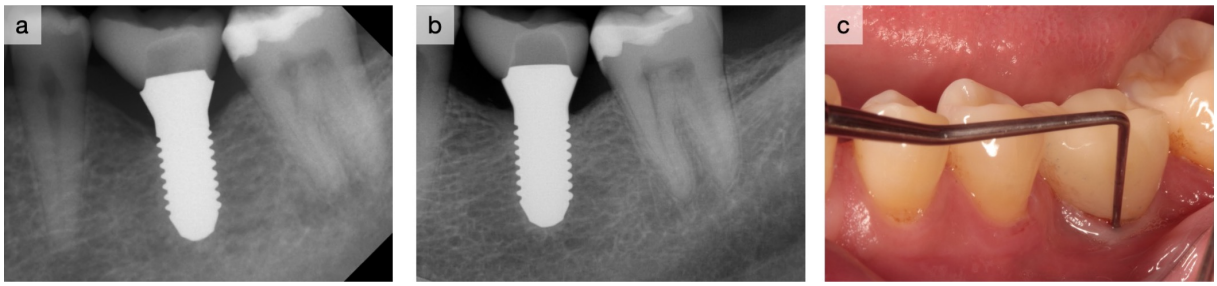

Figure 5: Case of mucositis at a two-piece zirconia implant after 9 years of follow-up: a) intraoral radiograph at 1-year follow-up; b) intraoral radiograph at 9-year follow-up where no bone loss could be detected; c) intraoral clinical image at 9-year follow-up, documenting the presence of BOP in absence of increased PD. (unpublished images of a patient of the zirconia implant clinical trial, Original work 5, kind courtesy Prof. J. Becker)

According to the recently published German S3 guideline on the use of dental ceramic implants (Thiem, Stephan et al. 2022), there is still limited clinical evidence showing a

reduced plaque accumulation and a related reduced risk of peri-implantitis with ceramic implants compared with titanium ones. A recent prospective cohort study comprising 16 patients with 32 implants (16 zirconia and 16 titanium) aimed at investigating host-derived parameters around the two implant types and natural teeth during the occurrence of experimental mucositis and subsequent recovery (Clever, Schlegel et al. 2019). The cessation of daily oral hygiene measures induced a stronger inflammatory response at the soft tissues around titanium as confirmed by interleukin-1β levels (Clever, Schlegel et al. 2019). Further, in the same cohort of patients, a significantly lower counts of *Prevotella intermedia* and *Tannerella forsythia* were found around teeth and zirconia implants compared with titanium implants (Clever, Schlegel et al. 2019).

These results were corroborated by a recent randomized clinical trial (RCT) in 42 patients with two neighbouring missing teeth replaced by one zirconia and one titanium implant (Bienz, Hilbe et al. 2021). Under experimental mucositis conditions, lower plaque and bleeding scores were found around zirconia implants. BOP significantly increased around titanium implants after three weeks of experimental mucositis induction, while values remained constant in the zirconia group.

As regards the therapy of peri-implant diseases, protocols derived from the treatment of titanium implants are commonly used. However, there is still scarcity of data on the success of these procedures when applied to zirconia implants.

One of the prerequisites of a successful treatment of peri-implantitis is the effective decontamination of the expose implant surface, without causing a concomitant detrimental effect of its integrity and biocompatibility. A recent systematic review investigated the impact of physical decontamination methods on zirconia implant surfaces (Tan, Khan et al. 2021). Results based on 11 included *in vitro* studies suggested the safety of air-abrasive devices with glycine powder, prophylaxis cups, and ultrasonic scalers with non-metal tips: By contrast, hand instruments and ultrasonic scalers with metal inserts might lead to zirconia surface damage. Diode lasers might also be suitable for surface decontamination.

For the treatment of peri-implant mucositis on zirconia implants, preliminary clinical results suggest the effectiveness of mechanical debridement using carbon curettes followed by local antiseptic therapy with CHX digluconate (Schwarz, John et al. 2015).

Preliminary clinical results suggest that the use of an Er:YAG laser may also be effective in the reduction of BOP in case of peri-implantitis on zirconia implants (Schwarz, John et al. 2015).

Future clinical studies are needed to investigate the effectiveness of different treatments for peri-implant mucositis and peri-implantitis affecting zirconia implants. Furthermore, specific treatments explicitly addressed to zirconia implants might also be developed.

PERSONAL WORKS

Original work 1: Implantoplasty: carbide burs vs diamond sonic tips. An in vitro study

Background

Implantoplasty is a common procedure performed during the surgical treatment of peri-implantitis consisting in smoothing the exposed contaminated implant surface, with the final goal of limiting the progression of peri-implantitis and the risk of recurrence. Among several tools, the most frequently reported are diamond or carbide burs followed by Arkansas stone or silicone polishers (Ramel, Lüssi et al. 2016, Costa-Berenguer, García-García et al. 2018, Stavropoulos, Bertl et al. 2019).

Despite its successful application, implantoplasty is not devoid of biological and mechanical complications (Stavropoulos, Bertl et al. 2019). Concerns include the risk of overheating, the dispersion of titanium particles that might sustain peri-implant inflammation and implant strength decline owing to the reduction of the implant diameter and structure (de Souza Júnior, Oliveira de Souza et al. 2016, Gehrke, Aramburú Júnior et al. 2016, Bressan, Ferroni et al. 2019).

For the successful treatment of peri-implantitis, it is crucial to obtain a smooth and less plaque-retentive implant surface. Implantoplasty should favour the reduction of implant surface roughness without compromising its biocompatibility. Low arithmetical mean roughness (Ra) values of 0.32 μm and 0.39 μm have been reached after implantoplasty with diamond burs followed by polishing or Arkansas burs, respectively (Ramel, Lüssi et al. 2016). Treatment with diamond burs followed by Arkansas was also demonstrated not to affect implant biocompatibility (Schwarz, John et al. 2017). However, the use of burs has been correlated to a decrease in the mechanical properties of the implants (Chan, Oh et al. 2013, Gehrke, Aramburú Júnior et al. 2016). As an alternative, the use of ultrasonic instruments with diamond-coated inserts has been described for implantoplasty with promising *in vitro* results (Raoofi, Sabzeghabaie et al. 2013). However, to the best of the authors' knowledge, the use

of sonic devices has not been previously investigated to this purpose. It was hypnotised that sonic device in combination with diamond-coated tips could represent a more conservative approach than rotational burs for implantoplasty, leading to comparable final surface roughness.

Therefore, the aim of this *in vitro* study was to compare implantoplasty performed with two different methods (i.e. with diamond sonic tips versus tungsten carbide egg-shaped burs, both followed by finishing Arkansas burs) in terms of treatment time, weight loss, surface roughness, implant wear, measured by means of micro-computed tomography (micro-CT), and fracture resistance.

Methods

A total of 18 titanium dental implants (4 mm diameter, 13 mm length) with external hexagonal connection and hybrid surface (Machined and Osseotite®, Zimmer Biomet, Palm Beach Gardens, FL, USA) were utilized. As shown in Figure 6, the most 6-mm coronal portion of the implants was subjected to implantoplasty either with a sequence of two tungsten carbide egg-shaped burs (BUR; n=6 implants) or with a sequence of two torpedo-shaped diamond sonic tip (SONIC; 6 implants), followed by finishing with Arkansas burs (BUR + A and SONIC + A, respectively). All the implants were treated by as single operator. To resemble the clinical conditions, implantoplasty was carried out till the implant surface appeared uniformly smooth and shiny. The remaining 6 implants were left untreated (CONTROL). The duration of the procedure was recorded.

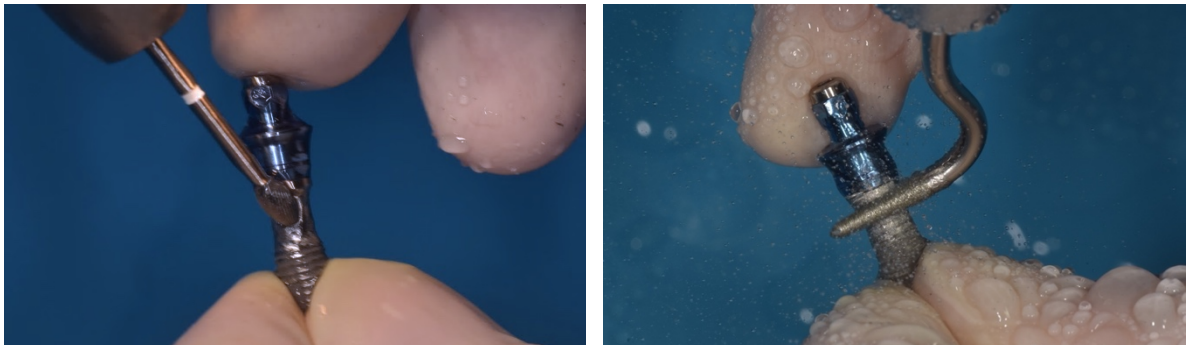

Figure 6: Implantoplasty performed with two different methods: a) tungsten carbide bur (left); b) diamond sonic tips (right). (own illustrations)

The flowchart summarizing the research design of the study is presented in Figure 7.

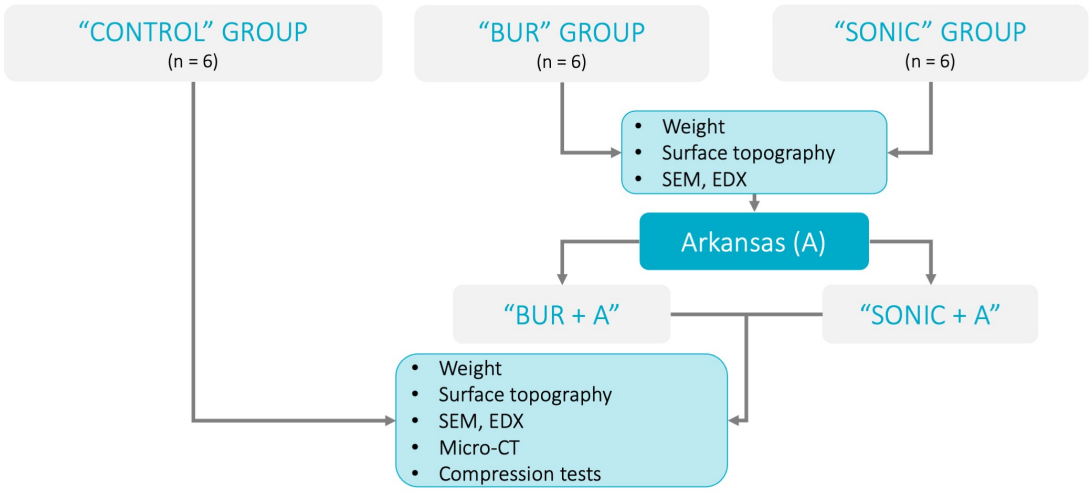

Figure 7: Flowchart of the research design employed in the study. (own illustration)

Briefly, the following experimental data was to collected:

1. Implant weight variation

For weight measures, a precision balance with a sensitivity of 0.001 g was used.

2. Surface topography analysis

High-resolution surface topography was conducted by means of a stylus profilometer. Implant surface texture was characterized using 2D profile roughness parameters, i.e. Ra (average roughness) and Rz (mean roughness depth). For representative 3D graphical images, 3D scanning of the surfaces was also performed.

3. SEM-EDS analysis

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) were applied for surface morphology and elemental characterization, respectively.

4. Micro-CT analysis

Micro-computed tomography (micro-CT) was used to evaluate volumetric material loss. In the 6-mm coronal portion of each implant 46 cross-sections parallel to the implant platform plane (plane 0) were obtained. Minimum cross-sectional area, position of the minimum cross-sectional area and mean cross-sectional after implatoplasty with respect to controls were analysed.

5. Compression tests

Static compression tests were carried out to assess implant fracture resistance.

Results and Discussion

Implantoplasty with sonic tips was significantly longer than with burs. The latter also led to a significantly higher implant weight loss than sonic instruments. The analysed 2D profile roughness parameters, i.e. Ra and Rz, were higher in the SONIC group than in the BUR, but equivalent final surface roughness values were recorded after polishing with Arkansas. After this step, the mean Ra values were lower than in the original Osseotite® surface (0.75 ± 0.07 µm) and amounted to 0.54 ± 0.06 µm and 0.60 ± 0.05 µm in BUR + A and SONIC + A, respectively.

At SEM analysis, both methods let to the removal of the implant threads and, in line with surface topography outcomes, the surfaces resulted similar after polishing with Arkansas. At EDS analysis, beside titanium, peaks of C and Al were detected after sonic and Arkansas treatments and might be due to the wearing of the instruments.

As regards micro-CT findings, sonic tips resulted to be more conservative than burs in terms of volume reduction, mean cross-sectional area and minimum cross-sectional area. Interestingly, the position of the latter was more apical in the BUR + A compared with both CONTROL and SONIC + A (Figure 8).

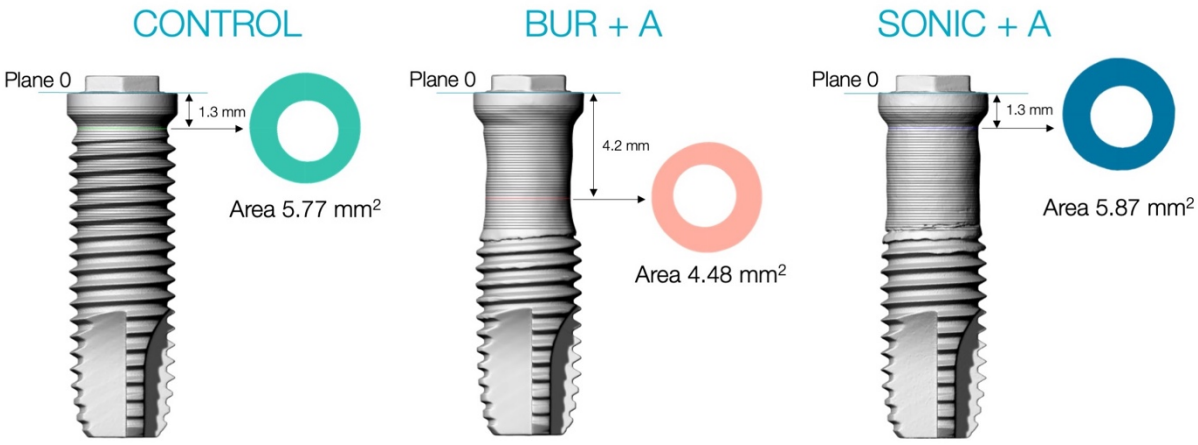

Figure 8: Minimum cross-sectional area: mean value and position in each group. (own illustration)

In accordance with other studies (Tribst, Dal Piva et al. 2017, Costa-Berenguer, García-García et al. 2018, Sahrmann, Luso et al. 2019), compression test revealed no statistical differences among the treatment groups and with respect to the controls. However, the study might have failed to detect any effect due to the limited number

of samples utilized. Furthermore, no correlation was found between maximum compressive force and micro-CT data.

In conclusion, within the limitations of this *in vitro* study, it can be affirmed that both methods allowed the achievement of a smooth implant surface after the final step with Arkansas. Implantoplasty with sonic diamond-coated tips resulted to be more conservative in terms of structure loss. However, the longer treatment time and the higher costs might restrict its application in every day practice. The beneficial effect of sonic tips might be clinically relevant in specific situations, such as for narrow-diameter implants, internal connection implants or in case of difficult access to the exposed implant threads by conventional rotary instruments.

Original work 2: Efficacy of 0.05% chlorhexidine and 0.05% cetylpyridinium chloride mouthwash to eliminate living bacteria on in situ collected biofilms: An in vitro study

Background

Periodontal and peri-implant diseased are frequent complex multifactorial pathologies (Derks and Tomasi 2015, Frencken, Sharma et al. 2017). There is strong evidence that these conditions are associated with plaque accumulation. This plays a role not only in the onset of these pathologies, but also in their progression and recurrence after treatment (Tonetti, Muller-Campanile et al. 1998, Renvert and Quirynen 2015, Müller Campanile, Megally et al. 2019, Bäumer, Toekan et al. 2020). To this aim antiseptic mouthwashes have been largely applied as adjunctive measures for the disruption of the biofilm on both teeth and implants. Among these products, CHX-based mouthwashes are the most frequently reported owing to their proved antimicrobial properties. Nevertheless, the prolonged usage of CHX has been associated to dose-dependent side effects (Smith, Moran et al. 1995, James, Worthington et al. 2017). As a consequence, CHX solutions at low concentration, combined with other antimicrobials, have been proposed with satisfactory clinical outcomes (Santos, Herrera et al. 2004, Escribano, Herrera et al. 2010). Among these products, cetylpyridinium chloride (CPC), is attracting increasing interest (Quirynen, Soers et al. 2005, Mor-Reinoso, Pascual et al. 2016, Pulcini, Bollaín et al. 2019, Bollain, Pulcini et al. 2021).

Therefore, the aim of this study was to investigate if a mouthwash with a low concentration of CHX and CPC was as effective as a conventional CHX mouthwash to eliminate living bacteria on *in situ* collected biofilms. In order to mimic the exposure of teeth and dental implants, hydroxyapatite (HA) and micro-rough titanium (Ti) disks were utilized.

Methods

The design of the study is illustrated in Figure 9.

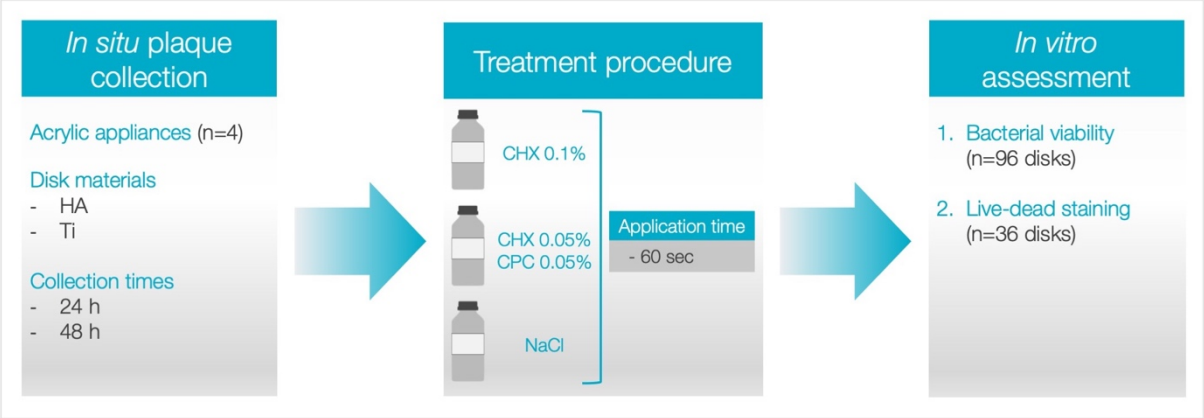

Figure 9: Study design. (own illustration)

Ti and HA disks were adapted to customized acrylic palatal appliances in order to accumulate biofilm *in situ*. Four healthy volunteers were recruited and randomly assigned to wear the device for 24 or 48 h (n=2 subjects per collection time). The devices were produced as in John et al. (John, Schwarz et al. 2015).

After plaque accumulation, the specimens were carefully removed and rinse with sterile water. The disks were randomly exposed for 60s to one of the following agents: 0.1% CHX (CHX 0.1), 0.05% CHX combined with 0.05% CPC (CHX + CPC), or sterile saline (NaCl) as negative control.

A total of 96 disks (n=8 disks per material, treatment and collection time) were used to quantify bacterial viability using a luminescent viability assay (BacTiter-Glo®, Promega, Madison, WI, USA).

Live-dead staining was carried out on 3 disks per material, treatment and collection time. The specimens were analysed using a stereomicroscope after staining with the LIVE/DEAD® BacLight™ solution (LIVE/DEADTM BacLight™, Thermo Fisher Scientific, Wesel, Germany).

Results and Discussion

For both surfaces (i.e. HA and Ti) and plaque collection times (i.e. 24 and 48 h), the highest bacterial viability values were observed in the NaCl group. Whereas, both CHX 0.1 and CHX + CPC presented comparable high antibacterial activity, as revealed by the low count per seconds measured in these groups and the absence of significant differences among them. When Ti disks were analysed, significant differences were detected between the NaCl and the two CHX-based solutions, after both 24 and 48h of plaque accumulation. Similar findings were observed in for HA disks. However,

significant failed between NaCl and CHX + CPC after 48 h of *in situ* plaque collection on HA surfaces, despite the absence of overlap between the respective interquartile ranges.

These findings are in line with the live-dead staining results. Indeed, for both collection time points, the experiment revealed almost no living cells on all disks after the application of CHX-based products. Whilst, on specimens treated with NaCl, the bacterial biofilm presented numerous living bacteria distributed on the entire surfaces.

This study supports the still limited but encouraging available evidence regarding the efficacy of mouthwashes containing low concentrations of CHX and CPS in the prevention, the treatment and supportive therapy of periodontal and peri-implant diseases (Quirynen, Soers et al. 2005, Pulcini, Bollaín et al. 2019, Bollain, Pulcini et al. 2021).

In summary, within the limitations of the present *in vitro* study, the test CHX + CPC mouthwash allowed to decrease the concentration of CHX while conserving high antibacterial activity. If this formulation is also accompanied by a reduced cytotoxic effect on different tissues remained to be demonstrated.

Original work 3: The effects of three chlorhexidine-based mouthwashes on human osteoblast-like SaOS-2 cells. An in vitro study

Background

A variety of dental implant decontamination methods has been proposed withing the surgical treatment of peri-implantitis, including the use of antiseptic mouthwashes (Schwarz, Schmucker et al. 2015). Chlorhexidine (CHX), in particular, has been frequently utilized to this aim owing to its renown antibacterial properties (Khoury, Keeve et al. 2019) (Daubert and Weinstein 2019), although discordant results have been reported on its beneficial effect on wound healing after different oral surgery procedures. It is still to be clarified if the direct exposure of the bone to antimicrobial agents after flap elevation might impair bone healing due to the potential tissue toxicity of the products. *In vitro* studies using osteoblasts or osteoblast-like cells, SaSO-2 cells, have reported on cell damage using 0.1% or 0.2% CHX (John, Becker et al. 2014, Vörös, Dobrindt et al. 2014). Another study revealed a dose- and time-depended impact of CHX on cell viability (Giannelli, Chellini et al. 2008).

In order to minimized the shortcomings of CHX-based mouthwashes, shorter exposure time and/or lower concentration of CHX alone or in combination with additional compounds, such as cetylpyridinium chloride (CPC), have been proposed. In the *Original work 2* described above (Becker, Brunello et al. 2021), contrary to NaCl, 0.05% CHX + 0.05% CPC and 0.1% CHX mouthwashes altered bacterial viability on *in situ* collected biofilms attached to micro-rough titanium surfaces with no significant differences among the groups after an exposure time of 60s. Taking into account the documented cytotoxic effect of CHX at higher concentration and the proved antibacterial activity of CHX at low concentration combined with CPC, aim of this study was to *in vitro* investigate the impact of three commercially available mouthwashes containing CHX at different concentrations, alone or in combination with CPC, on SaOS-2 cells in terms of cell viability, cytotoxicity and apoptosis.

Methods

Osteoblast-like cells (SaOS-2 cells) were seeded on sterile 96-well binding cell-culture plates following the protocol previously described in John et al. (John, Becker et al. 2014). The study design is summarized in Figure 10.

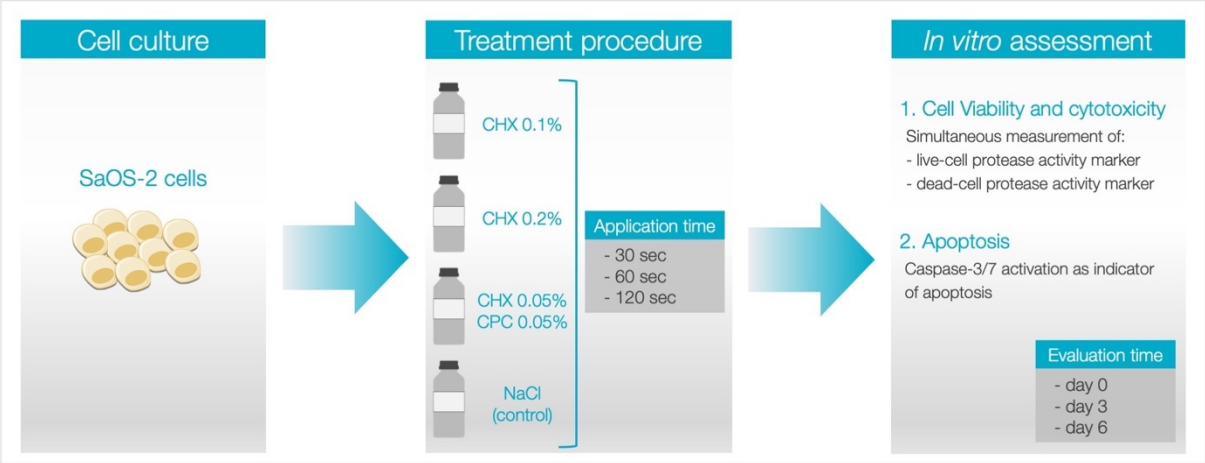

Figure 10: Study design. (own illustration)

After 3 days of culture, each well was randomly treated for either 30, 60 or 120 s with 0.1% CHX, 0.2% CHX, 0.05% CHX combined with 0.05% CPC (CHX + CPC), or sterile saline (NaCl) as control.

After cell exposure to test and control mouthwashes, the solutions were removed, the wells were gently rinsed with buffer solution and new culture medium was added. Finally, ApoTox-Glo™ Triplex Assay (Promega, Mannhein, Germany) was utilized to assess cell viability, cytotoxicity and apoptosis at three time points, i.e. at day 0 (after 2 hours), 3 and 6 after the exposure to the mouthwashes following the instruction of the manufacturer. For each application time and assessment time point, 8 wells per group were analysed (Figure 11). This triple assay allowed to simultaneously assess cell viability and cytotoxicity, by measuring two protease activities, one for living and one for dead cells respectively, owing to the different fluorescence emission spectra. Afterwards, as indicator of apoptosis, caspase-3/7 activity was examined on the same samples by adding a luminogenic caspase-3/7 substrate. All signals were measured using the same luminometer/fluorometer (Victor 2030, PerkinElmer, Rodgau, Germany).

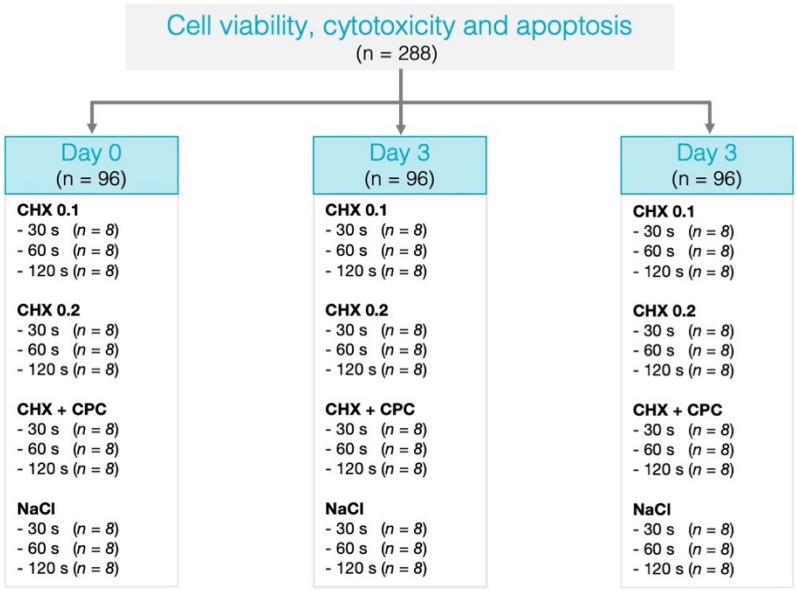

Figure 11: Well assignment for in vitro testing. (own illustration)

Results and Discussion

The highest cell viability values were registered in the saline group at all time points and for all application times, while the test mouthwashes affected SaOS-2 cells viability to a larger extent. All test groups presented decreasing cell viability values overtime, with no statistically significant differences between each other except at day 0. The application time was not relevant in the majority of the cases.

As regards cytotoxicity, at day 0 the highest values were registered with CHX 0.2, that resulted significantly more cytotoxic on SaOS-2 than CHX 0.1 (30s), CHX + CPC (all exposure times), and saline (60s and 120s). Contrary to the other test mouthwashes, the exposure time was found not to be relevant within the CHX 0.2 after two hours of culture. At both day 3 and 6 the highest values were observed in the control group. This could be explained by the early death of a broad range of cells once in contact with the test mouthwashes.

At all time points the highest apoptosis values were registered in presence of saline. This is in line with a previous study of our group (John, Becker et al. 2014), in which higher apoptotic values were recorded in presence of pure water as compared to CHX-based solutions. It could be assumed that the mouthwashes exert a predominant cytotoxic effect, while the high apoptotic values observed in the saline group might be caused by common environmental stresses, in particular after multiple days of culture (Krampe and Al-Rubeai 2010).

In summary, despite all test mouthwashes provoked irreversible cell damage *in vitro* as demonstrated by the findings at day 3 and 6, relevant differences among the mouthwashes were observed at day 0. At the early time point, the highest cytotoxic effect was noticed for CHX at high concentration, i.e. 0.2%, and shorter applications times were associated to lower cytotoxicity levels in both CHX 0.1 and CHX + CPC group. Finally, clinical trials should be performed to confirm the *in vitro* findings and to identify the ideal rinsing protocol for different oral surgery procedures, balancing the risks of cytotoxicity when the bone is exposed directly to the products and the required antimicrobial effect.

Original work 4: Effect of three chlorhexidine-based mouthwashes on human gingival fibroblasts: an in vitro study

Background

Chlorhexidine (CHX)-based mouthwashes have been largely employed as board-spectrum antiseptics for the prevention and treatment of periodontal and peri-implant diseases, as well as in the subsequent supportive therapy aiming at consolidated the obtained results. However, their use has been associated with dose-dependent adverse events, including tooth staining, transient taste disturbance and burning sensation (James, Worthington et al. 2017).

Particular circumstances may favour the onset of side effects, such as the usage of CHX over an extended time or the direct exposure of the connective tissues to the mouthwashes, for instance during postoperative wound healing owing to the absence of an intact epithelial barrier (Faria, Cardoso et al. 2009, Müller, Eick et al. 2017). A cost-effective method to reproduce connective tissue exposure to the antimicrobial agents consists in testing *in vitro* the response of human gingival fibroblasts (HGFs) to the products (John, Becker et al. 2014, Coelho, Laranjo et al. 2020, Alpaslan Yayli, Tunc et al. 2021).

In order to minimize the risk of side effects, low-concentration CHX mouthwashes eventually in combination with adjunctive agents, such as cetylpyridinium chloride (CPC), have been proposed (James, Worthington et al. 2017, Pulcini, Bollaín et al. 2019, Bollain, Pulcini et al. 2021). However, it is fundamental to maintained a balance between antimicrobial activity and cytotoxicity. Effective biofilm control had been previously demonstrated by our group using 0.05% CHX + 0.05% CPC and 0.1% CHX, as reported in the *Original work 2* of the present Habilitation thesis (Becker, Brunello et al. 2021).

Therefore, the aim of this study was to investigate *in vitro* the effects of three commercially available mouthwashes containing CHX at different concentrations, alone or in combination with CPC, on HGFs in terms of cell viability, cytotoxicity and apoptosis.

Methods

The design of the study is illustrated in Figure 12.

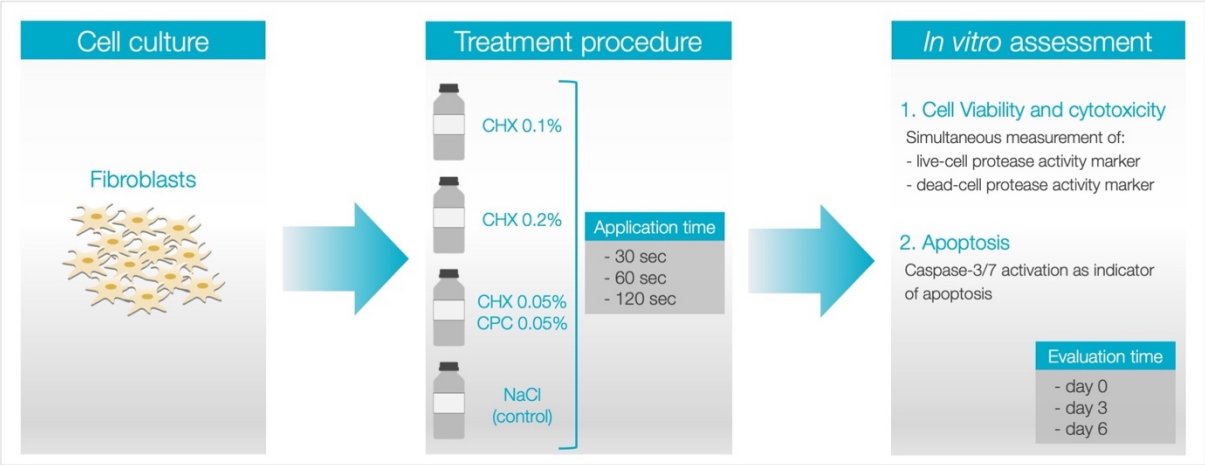

Figure 12: Study design. (own illustration)

Briefly, HGFs were seeded on sterile 96-well binding cell-culture plates as previously described in John et al. (John, Becker et al. 2014). A total of 288 wells were used for the experiments. Following 3 days of culture, the wells were randomly assigned to four treatment groups: 0.1% CHX, 0.2% CHX, 0.05% CHX combined with 0.05% CPC (CHX + CPC), or sterile saline (NaCl) as control. In each group cells were exposed to the solutions for either 30s, 60d and120 s. Afterwards the solutions were removed, the wells were gently rinsed with buffer solution and new culture medium was added. Cell viability, cytotoxicity and caspase-3/7 activity, as an indicator of apoptosis, were analysed using a single assay (ApoTox-Glo™ Triplex Assay, Promega, Mannhein, Germany) after 2 hours (day 0), 3 days and 6 days from cell exposure to the mouth-washes. Cell viability and cytotoxicity were simultaneously assessed by fluorometry measuring a live-cell and a dead-cell protease activity, respectively, due to the different emission spectra. Then, apoptosis was investigated using a luminogenic caspase-3/7 substrate.

Results and Discussion

Changes in all the investigated parameters were generally observed up to day 3 and values remained almost unchanged afterwards.

For all examination time points (i.e. 0, 3 and 6 days) and application times to the mouthwashes (30s, 60s, and 120s), the highest cell viability values were recorded in presence of saline and similar results were noted among the test groups. Interestingly, in most of the cases the treatment time did not affect cell viability.

On day 0, cellular toxicity was found to be influenced by the type of the mouthwash, its concentration and exposure time. The CHX 0.2 group presented the highest values, especially when the mouthwash was applied for 120s. In details, it resulted significantly more cytotoxic than the same product applied for a shorter time (30s), as well as NaCl and CHX + CPC applied for 120s. Furthermore, at the early examination time, i.e. day 0, no significant differences were identified between CHX + CPC and the control solution for all the exposure times.

As in other studies of our group using a similar experimental design (John, Becker et al. 2014, Brunello, Becker et al. 2021), at day 3 and 6 the saline group exhibited the highest cytotoxic effect. These findings could be explained assuming that most of the fibroblasts at these time points were already dead after the exposure to CHX-based agents.

As regards apoptosis, the highest values were registered in the control group at all time points. This is in line with what observed in the aforementioned studies (John, Becker et al. 2014, Brunello, Becker et al. 2021), and might be due to the predominant cytotoxic effect of the CHX-based mouthwashes over the apoptotic one.

In summary, the present findings suggest that CHX 0.2 has a higher cytotoxic profile compared to the other investigated products. Despite the difficulties in transferring the obtained *in vitro* data to the *in vivo* situation and clinical application, these observations could be clinically relevant. Therefore, caution might be used while using CHX at high concentration in certain circumstances, such as on open wounds.

Original work 5: Two-piece zirconia implants in the posterior mandible and maxilla: A cohort study with a follow-up period of 9years

Background

Zirconia dental implants are attracting increasing attention worldwide as an alternative to the widely used titanium implants for the replacement of missing teeth. They have recently gained popularity with the growing demand for aesthetics and metal-free solutions and their spread is expected to increase henceforth (Cionca, Hashim et al. 2017, Roehling, Schlegel et al. 2018, Sanz, Noguerol et al. 2019, Kohal and Dennison 2020).

Concerns regarding the structural weakness of ceramic implants determined the predominant development in early years of one-piece zirconia implants, whose prosthetic restorability could represent a major challenge in case of wrong three-dimensional (3D) positioning (Cionca, Hashim et al. 2017, Pieralli, Kohal et al. 2017). More recently two-piece zirconia implants, which possess higher restorative flexibility, were introduced in the market. Nevertheless, their late development reflects in the lack of long-term clinical studies (Cionca, Hashim et al. 2017, Pieralli, Kohal et al. 2017, Roehling, Schlegel et al. 2018).

A previous prospective cohort study conducted at the Department of Oral Surgery of the University Hospital of Düsseldorf evaluated the short-term clinical outcomes of two-piece zirconia implants placed in the posterior jaws supporting monolithic all-ceramic single crowns (Becker, John et al. 2017). The two-year findings were encouraging, with a cumulative survival rate of 95.8% (excluding early implant failures prior to loading), good soft tissue adaptation and rare mechanical and technical complications (Becker, John et al. 2017). Taking into account the importance of providing long-term data on two-piece zirconia implants, the present retrospective study was designed aiming at evaluate the clinical outcomes in the aforementioned patient cohort after 9 years of follow-up.

Methods

Sixty two-piece zirconia target implants were originally placed in the posterior jaws of 60 partially edentulous patients using a one-stage protocol (Figure 13) as reported in

Becker et al. (Becker, John et al. 2017). In 8 cases no primary stability was obtained. In the remaining 52 patients, the implants were restored with cemented fiberglass abutments (Figure 14) and monolithic all-ceramic single crowns (Figure 15).

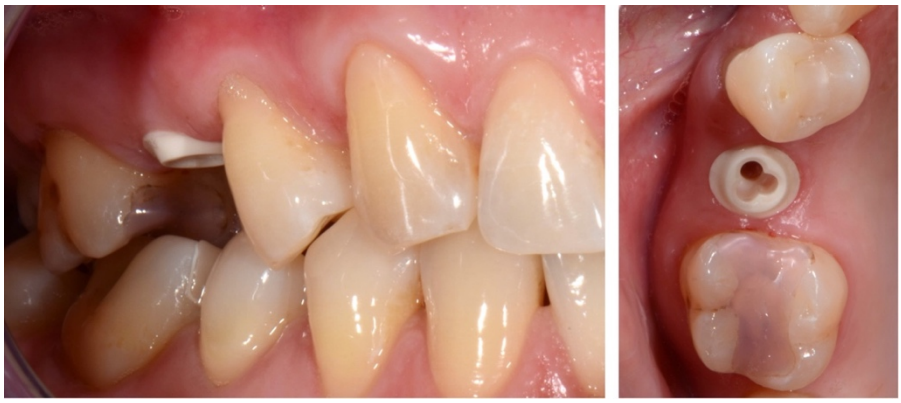

Figure 13: Transmucosal implant of a zirconia implant: a) lateral view (left); b) occlusal view (right). (unpublished images of a patient of the zirconia implant clinical trial, Original work 5, kind courtesy Prof. J. Becker)

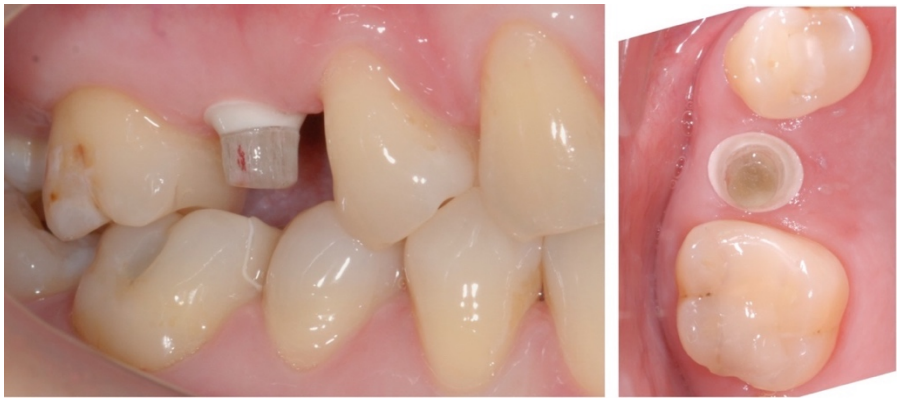

Figure 14: Fiberglass abutment cementation: a) lateral view (left); b) occlusal view (right). (unpublished images of a patient of the zirconia implant clinical trial, Original work 5, kind courtesy Prof. J. Becker)

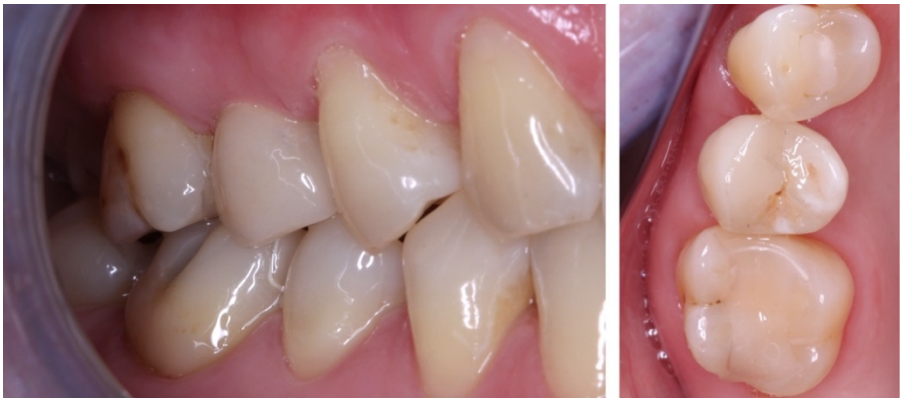

Figure 15: All-ceramic crown cementation: a) lateral view (left); b) occlusal view (right). (unpublished images of a patient of the zirconia implant clinical trial, Original work 5, kind courtesy Prof. J. Becker)

At the two-year follow-up (Becker, John et al. 2017), two implants were lost and four dropouts were registered. Therefore, 46 patients with one target implant each were recalled at the 9-year follow-up. Implant survival was recorded along with the following clinical parameters at implant level (i.e. plaque index–PI, bleeding on probing–BOP, probing depth–PD, and mucosal recession–MR), which were compared with previously collected data (baseline and 2 years). Technical (Heitz-Mayfield, Needleman et al. 2014) and mechanical complications were assessed, as well as the presence of peri-implantitis (Berglundh, Armitage et al. 2018) or mucositis (Renvert, Persson et al. 2018) at the target implant.

Results and Discussion

Out of 46 eligible patients, 30 responded. One implant failed after 110 months from insertion and data obtained from the remaining 29 patients was analysed.

Mean PI values at the target implants increased overtime, while no significant differences in mean BOP were detected between the three time points (i.e. baseline, 2 years and 9 years). It has to be noted that before the 2-year follow-up examination, out of the 29 target implants included in the present work, 10 implants were diagnosed with peri-implantitis and treated with Er:YAG therapy, as described in Schwarz et al. (Schwarz, John et al. 2015). At 9-year examination, these implants presented no significant differences in terms of BOP values as compared to the others.

PD values remained constant from 2- to 9-years follow-up and at the late time point the highest PD value was of 6 mm, that was recorded in two patients in only one site per implant.

As regards MR, the majority of the implants presented no recession at 9 years and no significant differences in mean MR values were observed between the three time points.

Three technical and six mechanical complications occurred between 2 and 9 years in 7 patients. Most of the complications involved the fiberglass abutment. Contrary to the short-term investigation (Becker, John et al. 2017), a high number of complications were recorded in the time lapse between the two studies, mostly involving the fiberglass abutment. All the complications were successfully solved replacing the prosthetic components. It is worth noting that no implant facture occurred.

Within the limitations of this study, which include the high number of dropouts, its retrospective design and the absence of a control group, an overall stability of the results between 2 and 9 years was observed. For the explored application, so as the replacement of single teeth in the posterior jaws, this two-piece zirconia implant system could represent a valid treatment option.

RÉSUMÉ AND OUTLOOK

The aims of the original works presented in this cumulative Habilitation thesis were to *in vitro* assess a new method for performing implantoplasty on titanium implants, to test different chlorhexidine (CHX)-mouthwashes used for the treatment of peri-implantitis in terms of antimicrobial activity and cytotoxicity, and, finally, to investigate the long-term clinical outcomes of zirconia dental implants.

In the section *Original work 1*: *Implantoplasty: carbide burs vs diamond sonic tips. An in vitro study* (Sivolella, Brunello et al. 2021), a novel procedure using sonic diamond tips for performing implantoplasty was presented. To the best of the author's knowledge, there is no other paper dedicated to this topic so far.
Implantoplasty is commonly performed during the surgical treatment of peri-implantitis, in combination with both resective or regenerative approaches. It consists in grinding the exposed threads and smoothing the implant surface, with the final goal of favouring the resolution of the pathology and decreasing the risk of recurrence (Khoury, Keeve et al. 2019, Stavropoulos, Bertl et al. 2019).
On one side this approach should improve the biological response to the treatment, on the other side it should not compromise the mechanical integrity of the implants. However, due to its subtractive nature, implantoplasty is deemed to weaken the implant strength and this could be particularly relevant in case of narrow-diameter implants (Chan, Oh et al. 2013, Gehrke, Aramburú Júnior et al. 2016, Costa-Berenguer, García-García et al. 2018). In a recent systematic review implantoplasty was reported not to be associated with any remarkable mechanical or biological complications on the short- to medium-term. However, owing to the limited data available, the authors underlined that the risk of mechanical complications could not be completely excluded (Stavropoulos, Bertl et al. 2019). As confirmed by micro-CT data, sonic diamond tips were found to be more conservative in terms of structure loss as compared to traditional burs. Micro-CT is commonly employed to test titanium biomedical components (Cobos, Norley et al. 2022). However, to the best of the author’s knowledge, it had not been previously applied to assess implant volumetric changes after implantoplasty. The non-destructive nature of this test allowed the subsequent assessment

of implant fracture resistance by static compression test on the same samples. No differences among the groups were found at compression test, even though the implants treated with burs tended to present lower mechanical resistance. The absence of statistically significance, however, might be ascribed to the reduced sample size.
Furthermore, the final step with Arkansas burs allowed to reach the same surface roughness in both sonic and bur groups, showing values in line with other studies (Ramel, Lüssi et al. 2016, Sahrmann, Luso et al. 2019, Beheshti Maal, Aanerød Ellingsen et al. 2020).
Despite more conservative, the utilization of sonic tips might be limited in daily practice by the longer treatment time, the high cost of the sonic tips and their rapid wear. Taking into account the fast deterioration of the inserts, it was decided a priori to change the set of inserts every other implant. However, it would be interesting to analyze the wear of the tips, the diminution of the cutting efficacy with their usage, and the potential increase of temperature generated during the procedure owing to wear. The detection of C peaks at EDS on implants treated with sonic tips further supports the release of diamond debris during implantoplasty.
Finally, the presence of the suprastructure during implantoplasty may affect the quality of the treatment, because the access to the defect might be impaired not only by the anatomy of the defect itself, but also by the prosthetic restoration (Khoury, Keeve et al. 2019). The need to remove the reconstruction could represent an issue especially in case of cement-retained solutions. It is likely that, to obtain the best results with the sonic tips, the prosthetic components should be removed, so that the tips could work parallel to the long axis of the implants. However, the model here utilized was not designed to investigate this aspect, that should be further explored *in vivo* o *in vitro* using models mimicking the clinical conditions, such as the presence of adjacent teeth.

As previously mentioned, peri-implant diseases are strictly correlated to the accumulation of plaque, therefore the removal of the biofilm is crucial in the prevention, treatment and maintenance of peri-implant mucositis and peri-implantitis. Among the adjunctive measures for biofilm removal, it is worth mentioning the use of antiseptic agents including CHX (Schwarz, Schmucker et al. 2015).

The *Original work 2* (Becker, Brunello et al. 2021), *3* (Brunello, Becker et al. 2021) and *4* (Brunello, Becker et al. 2022) consist in *in vitro* studies investigating different effects of CHX-based mouthwashes. To overcome the side effects of commonly used CHX mouthwashes, solutions containing reduced concentrations of CHX in combination of not with other active ingredients have been proposed (James, Worthington et al. 2017). In particular the studies aimed to evaluate if a commercially available mouthwash with CHX (0.05%) combined with CPC (0.05%) could be less cytotoxic than conventional CHX mouthwashes, while maintaining adequate antibacterial properties.

In the section *Original work 2: Efficacy of 0.05% chlorhexidine and 0.05% cetylpyridinium chloride mouthwash to eliminate living bacteria on in situ collected biofilms: An in vitro study* (Becker, Brunello et al. 2021), the efficacy of CHX-CPC was compared to that of a CHX (0.1%) mouthwash in reducing living cells in oral biofilms developed on hydroxyapatite and micro-rough titanium disks.
Biofilm formation on the disks was obtained by means of *in situ* plaque collection for 24 and 48 hours. Overall, as confirmed by both bacterial viability assay and live-dead staining, the two investigated CHX-based solutions demonstrated comparable antibacterial activity. Whereas, rinsing with saline was not effective against oral bacteria *in vitro*.
It was decided to investigate not only titanium, but also hydroxyapatite disks, as representative of dental implants and teeth, respectively. Indeed, despite the differences between periodontal and peri-implant pathologies in their progression pattern and in the biofilm composition, both inflammatory pathologies are associated with the presence biofilms (Kotsakis and Olmedo 2021).
The selection of *in situ* plaque collection could be considered a strength of this study, as it enables to mimic the normal biofilm growth, which is characterized by high complexity and a broad variety of bacterial strains (Verma, Garg et al. 2018, Abdullah, Al-Marzooq et al. 2019). Plaque collection was obtained in four periodontally healthy volunteers, who wore acrylic appliances containing the disks at a 1-mm distance from the palatal mucosa. However, the microbiota of these volunteers might differ from that of patients with a history of periodontal or peri-implant diseases (Lasserre, Brecx et al. 2018), who represent the target of the tested antiseptic agents. Future studies might consider a larger and more representative pool of patients for plaque collection.

In addition, it has to be noted that the short period up to 48 hours of biofilm growth on the disks might have not allowed substantial anaerobe growth, typical of periodontal and peri-implant pathologies (Siddiqui, Fidai et al. 2022).
A structured and functionalized biofilm comprises several microbes, including bacteria, as well as extracellular matrix (Bowen, Burne et al. 2018), and it is difficult to replicate its distinctive composition and organization *in vitro*. However, as alternative to *in situ* collection, other authors reported the successful use of different protocols for the *in vitro* cultivation of bacterial biofilm (de Avila, Avila-Campos et al. 2016, Roehling, Astasov-Frauenhoffer et al. 2017, Toma, Behets et al. 2018, Rigolin, Barbugli et al. 2019, Ichioka, Derks et al. 2022, Siddiqui, Fidai et al. 2022). Single-species models do not account for bacterial diversity typical of the oral cavity. Nonetheless, *in vitro* models permit the selection of multiple and specific bacterial strains. The *in vitro* approach can be implemented with salivary pellicle formation on the surfaces prior to biofilm development to simulate clinical conditions (de Avila, Avila-Campos et al. 2016, Roehling, Astasov-Frauenhoffer et al. 2017, Toma, Behets et al. 2018, Ichioka, Derks et al. 2022).

Taking into account the known cytotoxicity associated to CHX at high concentration, and the *in vitro* efficacy of the tested CHX+CPC formulation against oral bacteria, in the *Original work 3* (Brunello, Becker et al. 2021) and *4* (Brunello, Becker et al. 2022) of this comprehensive Habilitation thesis the author wanted to verify if this product was advantageous in terms of reduced cytotoxicity as compared to commercially available CHX mouthwashes at a concentration of 0.1% and 0.2%.

In the section *Original work 3: The effects of three chlorhexidine-based mouthwashes on human osteoblast-like SaOS-2 cells. An in vitro study* (Brunello, Becker et al. 2021) the effect of a CHX (0.05%) solution combined with CPC (0.05%) on osteoblasts was investigated. All the CHX-based mouthwashes affected SaOS-2 cell viability to a higher extend than saline. Findings suggest that the majority of the cells in contact with the test products died in the first days after the exposure and cell death was in these cases mainly the result of necrosis rather than apoptosis. The most relevant differences among the CHX-based agents were observed the same day of the exposure (day 0), with CHX 0.2% solution presenting the highest cytotoxic effect.

The osteoblasts were left exposed to the action of the solutions for three different times, i.e. 30, 60 and 120 seconds. These are reasonable application times, compatible with normal clinical procedures. It is unlikely that the direct contact of the mouthwash to the osteoblasts could last more than a few minutes during a surgical dental procedure. Moreover, similar exposure times were reported in other articles assessing the effect of different mouthwashes on osteoblasts or osteoblast like cells (John, Becker et al. 2014, Liu, Werner et al. 2018, Markel, Bou-Akl et al. 2021).

In order not to underestimate the regenerative capacity of the cells over time, the triple assay was not performed only at day 0, but longer observation times, i.e. day 3 and 6, were also selected as in a previous work from our group (John, Becker et al. 2014). Similar time points were chosen by Markel et al. (Markel, Bou-Akl et al. 2021), who evaluated the cytotoxicity and proliferation of the human osteoblast cells on day 3 and day 5 after treatment with different mouthwashes. In agreement with what reported here for CHX+CPC, a cytotoxic effect was detected also with CHX alone at low concentration (0.05%), with osteoblasts failing to recover over the course of 5 days (Markel, Bou-Akl et al. 2021).

To the best of the author's knowledge, this is the first work investigating the effect of a CHX (0.05%) + CPC (0.05%) solution on osteoblast-like cells *in vitro* and on this lies the originality of the study. As regards the study design itself, a conventional well-established monolayer cell culture was preferred over more complex 3D culture models, owing to its high reproducibility, the ease of use and the reduced costs. However, this represents the main limitation of the present study, since a 2D cell culture model cannot completely reproduce the exposure of bone tissue to the solutions. Indeed, osteoblasts reside within the mineralized bone tissue, that could alter the permeability and the adsorption of the antiseptic agents. Other factors could not be reproduced in this *in vitro* model, including the immunological response of the body and the dilution of the products in the saliva (Vörös, Dobrindt et al. 2014). It can be assumed that CHX 0.1% and CHX-CPC applied for limited time are preferrable over CHX 0.2% or longer exposure times, especially in case of intraoperative usage. Nonetheless, caution has to be taken when extrapolating these *in vitro* findings to *in vivo* conditions.

In the section *Original work 4: Effect of three chlorhexidine-based mouthwashes on human gingival fibroblasts: an in vitro study* (Brunello, Becker et al. 2022), the impact

of the same CHX-based mouthwashes tested in the *Original work 3* (Brunello, Becker et al. 2021) on fibroblasts was evaluated using the same study design.
As for SaOS-2 cells (Brunello, Becker et al. 2021), cell viability was higher in the saline group than in the test groups, that reported similar cell viability values among each other. The main difference among the test solutions was observed at day 0 in cytotoxicity levels, with the highest values measured after exposure to CHX 0.2%, especially after 120 s of application time.
For all the investigated parameters, i.e. cell viability, cytotoxicity, and apoptosis, the majority of the changes occurred between day 0 and day 3, while the values tended to steady thereafter. To better understand and estimated these dynamic processes especially in the early phases after treatment, the use of live cell imaging could be considered for future experiments (Isherwood, Timpson et al. 2011, Gelles and Chipuk 2016).
Also in this study, a monolayer cell culture method was chosen. Nevertheless, a 3D human oral mucosal model might be used in future studies. Despite the technical difficulties and costs related to the use of 3D models, they are supposed to better resemble the *in vivo* architecture of the tissues in which the cells reside (Moharamzadeh, Franklin et al. 2009, Langhans 2018, Jensen and Teng 2020, Klausner, Handa et al. 2021). Indeed, the oral mucosal is characterized by an epithelial outer layer, overlaying the connective tissue. In a 3D model the tissue permeability of a mouthwash through the epithelium could be better replicated (Klausner, Handa et al. 2021), avoiding the direct contact of the product to the fibroblasts. This would be particularly advantageous for increasing the transferability of *in vitro* data as regards the chronical usage of the antimicrobial mouthwashes in absence of open wounds.
Further, future clinical investigations should aim at evaluating not only the effect of various mouthwashes but also of different rinsing protocols on oral mucosal health, postoperative tissue healing and periodontal and peri-implant disease control,

In the section *Original work 5: Two-piece zirconia implants in the posterior mandible and maxilla: a cohort study with a follow-up period of 9 years* (Brunello, Rauch et al. 2022), the long-term clinical results of two-piece zirconia implants were presented. This study significantly contributes to the limited body of knowledge in this field. Indeed, as stated in the most recent German guidelines about the use of ceramic dental

implants (Thiem, Stephan et al. 2022), there is a "lack of reliable long-term data, especially in the case of two-piece implant systems".

Only a few papers reporting medium-term data are available at the moment, with a relative low number of implants per study. In a randomized pilot trial comparing two-piece zirconia implants supporting single crowns versus titanium implants, similar clinical outcomes were obtained in the two groups at 80-month follow-up (Koller, Steyer et al. 2020). Nonetheless, the results should be interpreted with cautions due to the limited samples size. Indeed, 28 implants (i.e. 14 per group) in 21 patients could be evaluated, since 2 zirconia and 1 titanium implants were lost in the meantime.

The medium-term outcomes of two-piece zirconia implants supporting full-ceramic crowns are also reported in a prospective cohort study (Cionca, Hashim et al. 2021). At the 6-year follow-up 39 implants (out of the initial 49) were available for examination in 24 patients regularly attending the maintenance visits. In the *Original work 5* here discussed, 30 patients with one target implant each attended the 9-year recall visit. Compared to Cionca et al. (Cionca, Hashim et al. 2021), a higher number of dropouts was recorded, accounting for approximately one third of the eligible patients. However, no significant differences were found after 2 years of follow-up in terms of any of the clinical variables considered between the cohort of patients examined at 9 years and the dropouts. Hence, it is likely that the study population truthfully represented the original cohort of patients (Becker, John et al. 2017) in terms of compliance and clinical conditions.

It is worth noting that the zirconia implant systems tested in the two aforementioned studies of other groups are no longer produced (Koller, Steyer et al. 2020, Cionca, Hashim et al. 2021). Whilst, the implant system utilized in the *Original work 5* of this comprehensive Habilitation thesis is still available in the market, and this increases the transferability of the reported data.

As regards signs of peri-implant inflammation, BOP and PD are deemed to be fundamental indexes for the detection of peri-implant inflammation and for the monitoring its progression. No BOP was found at the majority of the target implants and mean BOP as well as PD remained constant between the 2- and 9-year follow-up, confirming the stability of the results overtime.

Although clinical and animal studies are suggesting a reduced risk of peri-implantitis with zirconia implants compared to titanium ones (Clever, Schlegel et al. 2019,

Roehling, Gahlert et al. 2019, Bienz, Hilbe et al. 2021), the available evidence is still scarce to draw final conclusions (Thiem, Stephan et al. 2022). Since 10 target implants examined at 9 years had been diagnosed with peri-implantitis and treated with Er:YAG (erbium-doped: yttrium, aluminium and garnet) laser before the 2-year recall (Schwarz, John et al. 2015), we wanted to investigate if there was any significant differences in mean BOP at 9 years between implants previously treated for peri-implantitis and the remaining ones. Interestingly, Kruskal-Wallis test revealed no significant differences. Looking back at the supportive care protocol adopted in our Department, the early detection and treatment of these cases might have played a crucial role in the successful resolution of these complications. In this context, patients' awareness about the importance daily home maintenance and of lifelong individualized professional recall regimen is crucial (Brunello, Gervasi et al. 2020).

Most of the complications observed between 2 and 9 years of follow-up consisted in abutment fractures. These occurred after a mean observation time of 53.7 months (SD 22.9). This is in line with what reported in the 6-year prospective cohort study of Cionca et al. (Cionca, Hashim et al. 2021). They observed fractures of the abutment in six cases (out of 39 included implants) in six distinct patients, all at least 4 years after loading. Despite they used another two-piece design implant system, all the abutment fractures occurred at the level of the implant platform as in the current study. Similarly, all the cases were successfully solved with the removal of the fractured portion and with the replacement of the prosthetic restoration. Furthermore, they recorded one implant fracture (Cionca, Hashim et al. 2021), whilst in the present investigation this complication never occurred, despite the implants had been placed in the posterior jaws, where the masticatory forces are higher.

Overall, this two-piece zirconia implant system could represent a valid treatment option for the replacement of single teeth, while future studies should confirm its suitability for other clinical applications.

To conclude, the works presented in this cumulative Habilitation thesis demonstrate various mechanical and chemical methods for implant surface decontamination in cases of peri-implantitis. The methods include the use of diamond-coated sonic tips, originally designed for prosthetic applications, to perform implantoplasty. This ap-

proach resulted *in vitro* to be more conservative as compared to commonly used traditional burs. However, the high cost of the inserts and the longer operative time could discourage the clinicians to adopt this technique in their daily activity.
The sonic inserts available on the market at the moment quickly lose their cutting efficacy. From a commercial perspective, there is the need to develop inserts with improved wear resistance at a reasonable price. The design of the inserts could also be optimized for this specific application, thus increasing the accessibility of the insert to the exposed implant surface without the need to remove the superstructure.
Furthermore, the mechanical integrity of the implants seems to be preserved with this approach, and this could be particularly relevant in case of narrow-diameter implants. This should be further investigated *in vitro* by means of static and dynamic mechanical tests on implants presenting different designs, using a sufficiently large sample size to answer the research question of interest.
Further, RCTs should be conducted to assess the long-term therapeutic resolution of the peri-implantitis and the occurrence of mechanical complications (i.e. implant fractures) after different implantoplasty procedures in combination with both non-regenerative and regenerative surgical treatments of peri-implantitis.

Implant surface decontamination can also be obtained with chemical products. In the present cumulative Habilitation thesis, the *in vitro* antibacterial efficacy and cytotoxicity of CHX-based mouthwashes was extensively investigated. Taking into consideration the side effects of CHX at high concentration, the final goal of the research was to elucidate if a combination of CHX and CPC at low concentrations could be advantageously used for the treatment of peri-implant diseases.
The product resulted to be effective against oral bacteria grown on moderately rough titanium implant surfaces. Future *in vitro* studies should be conducted using other dental implant materials, such as zirconia. Further, since the investigated low dose CHX-CPC mouthwash is particularly indicated for the prevention of periodontal and peri-implant disease recurrence, it would be interesting to evaluate its effect not only on potentially exposed implant surfaces, but also on materials commonly used for the production of implant abutments, such as machined titanium, zirconia or polyetherketoneketone.

Clinical studies should be conducted to confirm the *in vitro* data and to identify specific rinsing protocols, in terms of rinsing time and frequency, depending on the selected clinical application, with the final goal to achieve an adequate antibacterial activity in absence of undesired side effects.

Finally, the last work here presented reported the clinical performances of two-piece zirconia implants after 9 years of follow-up. Cases of peri-implant mucositis and peri-implantitis occurred along the clinical study. The first were treated with mechanical debridement and local application of chlorhexidine digluconate, while the latter were treated with Er:YAG laser therapy. Despite the success of the proposed treatments, future RCTs should be conducted to investigate the efficacy of different protocols for zirconia implant surface decontamination in different clinical scenarios. Furthermore, considering the scarcity of data on zirconia implants compared with titanium ones, future studies should address the influence of different decontamination methods on the properties of zirconia implant surfaces, in terms for instance of superficial chemical composition or roughness modifications.

LIST OF ABBREVIATIONS

BOP	Bleeding on probing
CA	Commercially available
CHX	Chlorhexidine
CI	Confidence interval
CPC	Cetylpyridinium chloride
CT	Computed tomography
EDS	Energy dispersive X-ray spectroscopy
Er:YAG	Erbium-doped: yttrium, aluminium and garnet
HA	Hydroxyapatite
MR	mucosal recession
NCA	Non-commercially available
PD	Probing depth
PI	Plaque index
RCT	Randomized clinical trial
SEM	Scanning electron microscopy
Ti	Titanium
2D/3D	Two- / three-dimensional

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1.Original work

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ORIGINAL RESEARCH

CLINICAL ORAL IMPLANTS RESEARCH WILEY

Implantoplasty: Carbide burs vs diamond sonic tips. An in vitro study

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Abstract

Objectives: Implantoplasty (IP) is a treatment option for peri-implantitis. Mechanical concerns were raised on fracture resistance of implants subjected to this procedure. This study aimed to compare two methods of IP in terms of implant wear and fracture resistance, and of surface topography.

Material and methods: Eighteen cylindrical screw-shaped dental implants (4 mm diameter, 13 mm length) with an external hexagonal connection were used. IP was performed on the first 6-mm implant surface with a sequence of burs or diamond sonic tips, both followed by an Arkansas finishing. IP duration and implant weight variation were recorded. Micro-computed tomography (micro-CT) was used to evaluate material loss. Implant fracture resistance was assessed by static compression test. Surface topography analysis was performed with a stylus profilometer. Scanning electron microscopy–energy dispersive X-ray spectroscopy (*SEM*-EDS) was applied for implant surface morphology and elemental characterization.

Results: Micro-CT showed less material loss in sonic compared to burs. No statistically significant difference was found between the mean fracture resistance values reached in bur and sonic, both followed by Arkansas, and with respect to control. IP performed with burs led to a smoother surface compared to sonic. Equivalent final surface roughness was found after Arkansas in both IP procedures. *SEM*-EDS showed a deburring effect associated to sonic and revealed carbon and aluminum peaks attributable to contamination with sonic diamond tips and Arkansas bur, respectively.

Conclusions: IP with sonic diamond tips was found to be more conservative in terms of structure loss. This could have a clinical relevance in case of narrow-diameter implants.

KEYWORDS

bone implant interactions, CT imaging, surface chemistry

1 | INTRODUCTION

Peri-implantitis is described as a pathological condition involving peri-implant tissues, featured by inflammation of the peri-implant mucosa and progressive loss of the supporting bone (Schwarz et al., 2018). Surgical and non-surgical therapies have been proposed for its treatment (Khoury et al., 2019; Renvert et al., 2008).

Implantoplasty (IP) can be performed at the time of surgical peri-implantitis treatment. IP consists in wearing the exposed implant threads with the aim of polishing and smoothing implant surface to reduce bacterial recolonization (Bürgers et al., 2010; Toma et al., 2018). IP, usually done with a sequence of diamond or carbide burs followed by Arkansas stone or silicone polishers, has been proven to limit peri-implantitis progression, thus leading to an improvement of clinical and radiological parameters (Bianchini et al., 2019; Costa-Berenguer et al., 2018; Matarasso et al., 2014; Ramel et al., 2016; Romeo et al., 2005, 2007; Stavropoulos et al., 2019).

Peri-implant bone defect morphology can limit the angulation of a bur and, in turn, affect the outcome of IP. Concerns have also been raised regarding the biological and mechanical complications associated with IP. Overheating of the implant and the surrounding bone can occur during IP; however, this drawback can be overcome when proper irrigation is used (De Souza Júnior et al., 2016; Raoofi et al., 2013; Sharon et al., 2013). The role of titanium (Ti) debris dispersion in hard and soft peri-implant tissues, which might sustain the inflammatory process, is still controversial (Bressan et al., 2019; Kumazawa et al., 2002; Noronha Oliveira et al., 2018; Safioti et al., 2017; Suárez-López Del Amo et al., 2018). Although there is no definitive evidence that the presence of Ti particles in peri-implant tissues is correlated to peri-implantitis, this statement cannot be excluded (Stavropoulos et al., 2019). Finally, some studies report on ultramicroscopic morphology and elemental implant surface composition after IP (Beheshti Maal et al., 2020; Schwarz et al., 2017), however a definitive correlation with hard and soft tissue healing in humans cannot be drawn.

Mechanical complications might be caused by a reduction in the implant diameter and wall thickness after IP (Chan et al., 2013; Gehrke et al., 2016; Tribst et al., 2017).

It has been reported that implant surface roughness above a threshold value Ra of 0.2 μm is directly proportional to plaque colonization (Albouy et al., 2011; Renvert et al., 2011). IP modifies implant surface topography. Arithmetical mean roughness (Ra) values ranging between 0.32 μm and 1.67 μm have been reached after IP, with the lowest value obtained with a sequence of diamond burs and silicone polishers (Costa-Berenguer et al., 2018; Ramel et al., 2016; Raoofi et al., 2013; Toma et al., 2018).

The use of ultrasonic instruments has been described for IP with encouraging results (Raoofi et al., 2013). Sonic scalers are air-powered units that operate at low frequencies ranging in 3–8 kHz (Arabaci et al., 2007). Various tips are available, depending on the purpose (e.g., restorative, prosthetic, bone surgery). Diamond-coated tips are indicated for odonto- and osteoplasty. Sono-abrasive technique is well known in the field of minimum intervention dentistry. It is considered alternative and complementary to rotary instrumentation for the selective preparation and finishing procedures of enamel and dentinal tissues with excellent relationships between efficacy, quality, and safety (Decup & Lasfargues, 2014). The risk of iatrogenic damage to adjacent teeth is also reduced (Opdam et al., 2002).

In implant and bone reconstructive surgery, sonic is an alternative osteotomy method and can be used as a substitute to the conventional bur method (Heinemann et al., 2012; Viganò et al., 2015). The use of sonic devices has not been reported so far for IP.

The hypothesis is that the use of sonic devices with adequate tips is a safer and more conservative method than burs for IP, with similar final surface roughness.

The primary aim of this study was to compare the weakest dental implant section, measured by means of micro-computed tomography (micro-CT), after IP with diamond sonic tips versus tungsten carbide egg-shaped burs, both in combination with finishing Arkansas burs.

The secondary aims were to compare IP performed with the two methods in terms of treatment time, weight loss, surface roughness, and fracture resistance. The presence of an association between fracture resistance and micro-CT data was also investigated.

2 | MATERIAL AND METHODS

This study was reported in accordance to the modified Consolidated Standards of Reporting Trials (CONSORT) guidelines for reporting in vitro studies on dental materials (Faggion, 2012) (Appendix S1). Ethics approval was not required for this in vitro study.

2.1 | Implants

Eighteen cylindrical screw-shaped cpTi grade IV dental implants, 4 mm in diameter and 13 mm in length, with an external hexagonal connection (Osseotite® and machined hybrid design, Zimmer Biomet, Palm Beach Gardens, FL, USA). The most coronal 6 mm portion was subjected to IP with rotating burs (6 implants) or sonic tips (6 implants). The remaining 6 implants were used as controls.

2.2 | Implantoplasty procedures

Implants were subjected to IP using two different methods: a) a sequence of two tungsten carbide egg-shaped burs (H379.310.023 and H379UF.310.023, Komet Dental, Lemgo, Germany) (Figure A1 a) with decreasing toothing (BUR); and b) a sequence of two torpedo-shaped diamond sonic tips (SF878K.000.018 and SF8878K.000.018, Komet Dental) (Figure A1 b) attached to an air scaler (SF1LM, Komet Dental) (SONIC). Both groups were then treated by finishing with Arkansas burs (Dura-White Stones FL2 FG 0,244, Shofu, Kyoto, Japan) (BUR + A and SONIC + A, respectively) (Figure A1 c).

The IP was performed by one expert clinician (SS) under loupe magnification until the 6-mm coronal portion of the implant presented a uniform visually smooth and shiny surface, as already reported (Costa-Berenguer et al., 2018; Ramel et al., 2016; Sahrmann et al., 2019; Toma et al., 2016). The applied pressure was not standardized, to increase the external validity of this in vitro study. The duration of the procedures was recorded by an external examiner (FM). A new set of burs or tips was used for every other implant. After IP, the implants were cleaned by irrigation with distilled water and dried with compressed air.

2.3 | Implant weight variation

A precision balance with a sensitivity of 0.001 g (Sartorius Basic, Sartorius AG, Germany) was used for measuring implant weight before IP and after each step (BUR, SONIC, BUR + A, and SONIC + A).

2.4 | Surface topography analyses

High-resolution surface topography was analyzed using a stylus profilometer (Form Talysurf i-Series, Taylor Hobson Ltd, Leicester, UK), with a 2 μm stylus tip radius. For 2D roughness profile analysis of each control, a 2.2 mm x 1 mm area in the unthreaded apical rough portion (OSSEOTITE®) and a 2 mm x 0.4 mm area in the smooth collar (MACHINED) were obtained. Four areas of 2.2 mm x 1 mm were scanned in the treated portion of test implants, turning them by 90 degrees every scan, both before (BUR and SONIC) and after polishing with Arkansas burs (BUR + A and SONIC + A).

The surface texture was defined using the following profile roughness parameters: Ra (average roughness) and Rz (mean roughness depth).

The profile data were filtered applying a Gaussian filter with a sampling length equal to 0.25 mm to eliminate waviness and form components, in accordance with ISO 4,288 and ISO 25,178.

Data analysis was performed using Talymap software (Taylor Hobson Ltd, Leicester, UK).

3D scanning of 0.5 mm x 0.5 mm areas using a 0.01 mm increment was performed, to obtain a 3D graphical representation of each surface.

2.5 | *SEM*-EDS analysis

Surface morphology and elemental composition of control and treated implants were analyzed by scanning electron microscopy (FEI ESEM Quanta 200, FEI Company, Hillsboro, OR) and energy dispersive spectroscopy (EDS).

2.6 | Micro-CT analysis

The quantitative determination of geometric variation of the implants after IP was analyzed using a micro-CT system Diondo d2 (Diondo GMBH, Hattingen, Germany). Six implants per group (control, BUR + A and SONIC + A) underwent micro-CT scanning. The parameters used for CT scanning were as follows: voltage 200 kV, current 220 mA, exposure time 0.5 s, voxel size 18 μm. A 0.5 mm copper filter was used to reduce artifacts.

After 3D image reconstruction, for generating STL (STereo Lithography interface format) files of the implants VGStudio MAX 2.1 (Volume Graphics GmbH, Heidelberg, Germany) was used.

A standard surface determination with a fixed gray scale value of 32,543.45 was used to extract the STL files from micro-CT volume reconstruction.

These STL files were then imported in Geomagic Studio Qualify 12 (Geomagic®, Morrisville, NC, USA) to perform mesh analysis. One STL file of control group was chosen as the master for building a reference coordinate system and aligning all the other models with it.

After that, STL models were imported in a computer-aided design (CAD) software, Rhinoceros 6 (Robert McNeel & Associates, Seattle, WA, US). To compare the two IP methods, implant volume calculation on control and tested samples was performed.

Forty-six cross-sections parallel to the implant platform plane (plane 0) of each implant were obtained. The mean and the minimum cross-sectional areas of the 46 cross-sections were identified for each implant. From the minimum cross-sectional area, the position along the long axis was obtained.

2.7 | Compression tests

All the implants (6 control, 6 BUR + A and 6 SONIC + A) were subjected to a fracture compression test, in accordance with the specifications of the standard ISO 14,801:2016.

A servo-hydraulic test machine equipped with a 3 kN load cell (MTS Acumen 3 Electrodynamic Test System, MTS Systems Corporation, Eden Prairie, MN, USA) was used.

A 6 mm horizontal bone resorption was simulated placing each implant orthogonally into a resin block with a modulus of elasticity over 3GPa (AcryOrt SC, Ruthinium Group, Rovigo, Italia).

An 8 mm healing abutment (THA58 EP®, Zimmer Biomet Dental) was then placed on each implant with a torque of 20 Ncm by means of a torque ratchet as specified in the manufacturer's recommendations.

The samples were placed on a stainless steel clamping jaw to obtain a 30° angle between the longitudinal axis of the implant and the loading direction of the testing machine and a constant speed of 1 mm/min was set.

For data collection, MTS Testsuite software (MTS Systems Corporation) was used.

The maximum compressive force (F_{max}) was measured as the maximum force reached before a 150N decrease due to sample failure.

2.8 | Statistical analysis

The sample size for this study was calculated a priori according to the expected difference in the primary outcome measure (the minimum cross-sectional area) between BUR + A and SONIC + A groups. We assumed that a mean difference of 1.5 *SD* in the minimum cross-sectional area between the two groups would have been plausible. With a power of 80% and a type I error of 5%, a sample size of 12 implants (6 BUR + A implants and 6 SONIC + A implants) was required to detect a standardized effect size of 1.5 in the difference of the minimum cross-sectional area between BUR + A and SONIC + A groups. Moreover, 6 additional implants were included as a control group for further comparisons.

Continuous data were expressed as mean and standard deviation (*SD*). Comparisons between BUR and SONIC groups were performed

using paired Student *t* test. BUR and SONIC groups were also compared with control group using paired Student *t* test. Paired data were assumed since all implants used in the present study were commercially available implants, characterized by the same macro- and micro-design and coming from the same batch.

Adjustment for multiple tests was not performed given the small sample size and the exploratory (not confirmatory) nature of the study. All tests were 2-sided and a *p*-value below .05 was considered statistically significant. Data analysis was performed using R 3.5 (R Foundation for Statistical Computing, Vienna, Austria).

3 | RESULTS

Details on the adherence to the modified CONSORT criteria are reported in Appendix S2.

3.1 | Time

The mean grinding time was 284 s (*SD* 50) and 658 s (*SD* 65) for BUR and SONIC, respectively ($p < .0001$). The mean time after polishing with Arkansas burs was 453 s (*SD* 44) for BUR + A and 860 s (*SD* 68) for SONIC + A ($p < .0001$).

3.2 | Implant weight

Details of implant weight are reported in Table A1.

Implant weight decrease from pre-IP to post-IP was higher in BUR versus SONIC ($p < .0001$), while no statistically significant difference was found in implant weight reduction after Arkansas treatment ($p = .99$).

3.3 | Surface topography analyses

Ra was lower in BUR than in SONIC ($p = .006$), whereas no significant differenced were found between BUR + A and SONIC + A ($p = .15$).

TABLE 1 Surface topography characterization: Ra and Rz values, in μm

Surface	Ra mean (*SD*)[a]	Rz mean (*SD*)[a]
MACHINED	0.06 (0.01)	0.42 (0.12)
OSSEOTITE®	0.75 (0.07)	4.24 (0.35)
BUR	0.64 (0.15)	3.82 (0.45)
BUR + A	0.54 (0.06)	2.78 (0.30)
SONIC	0.91 (0.16)	4.84 (0.93)
SONIC + A	0.60 (0.05)	3.03 (0.34)

Abbreviations: Ra, average roughness; Rz, mean roughness depth; *SD*, standard deviation.

[a]Cutoff filter λc = 0.25 mm.

Similarly, the Rz was lower in BUR versus SONIC ($p = .01$), but not in BUR + A vs SONIC + A ($p = .23$).

No statistically significant difference in Ra values was found between BUR and BUR + A ($p = .13$), while the Rz was higher in BUR

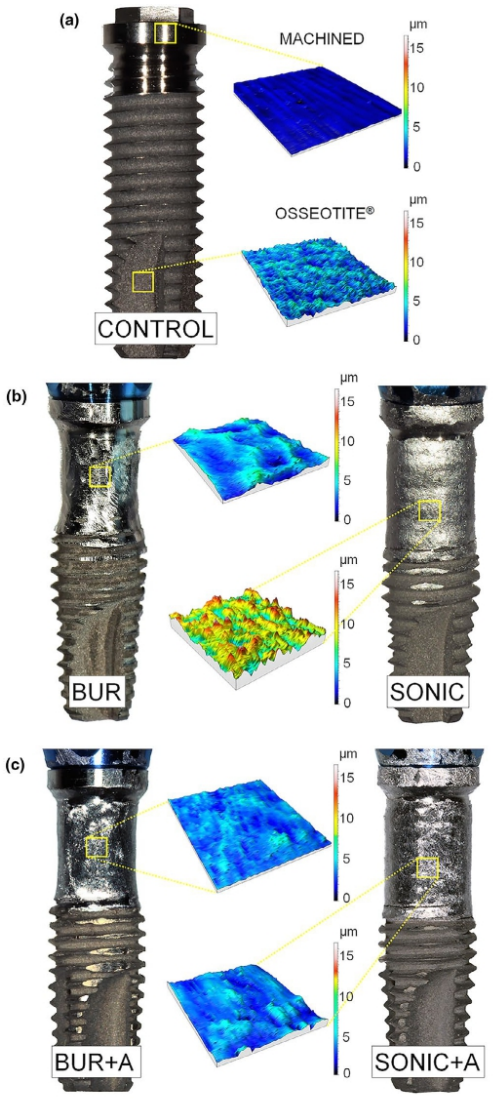

FIGURE 1 Macroscopic implant images and 3D maps of regions of interest (in yellow): (a) control implant, characterized by MACHINED and OSSEOTITE® surfaces; (b) test implants after using the tungsten carbide burs (BUR), on the left, and torpedo-shaped sonic tips (SONIC), on the right; and (c) and after using the Arkansas bur, that is, BUR + A (left) and SONIC + A (right)

than in BUR + A (p = .02). Ra (p = .03) and Rz (p = .03) values were higher in SONIC than in SONIC + A group.

The Ra and Rz values for each group are provided in Table 1.

3D maps of each investigated surface are reported in Figure 1.

3.4 | *SEM*-EDS analysis

SEM images are presented in Figures 2 and 3, and Figure A2.

SEM images demonstrated that the effect of the burs was the creation of a more irregular surface, with grooves, notches, and channels. After sonic treatment, implants maintained the original morphology to a greater extent and it was characterized by the alternation of threads crests and roots. Moreover, the surface appeared rippled as well, but more smeared, in particular in the most apical area.

Metal burrs were reduced in both groups after Arkansas. The bur's flattening effect in BUR + A was maintained, while the transition between thread crest and roots was less evident in SONIC + A.

EDS analysis of control implant surfaces revealed high purity of Ti. After IP with burs only (BUR), a similar EDS spectrum was found. Carbon (C) peaks were observed both on SONIC and SONIC + A treated surfaces. After polishing with the Arkansas bur in both groups the surface was composed of pure Ti, and an aluminum (Al) peak was found in correspondence of some dark spots. Details of EDS analysis are presented in Figure A3.

3.5 | Micro-CT analysis

Micro-CT data and images are reported in Table 2 and Figure 4, respectively.

Implant volume was smaller in BUR + A than in SONIC + A (p = .002), and both were smaller with respect to controls (p = .02 and p = .01, respectively) (Figure 5a).

Minimum cross-sectional area was smaller in BUR + A than in SONIC + A (p = .003), while it was not statistically different in BUR + A vs. controls (p = .08) and SONIC + A vs. controls (p = .13) (Figure 5b).

The mean position of the minimum cross-sectional area was found similar in controls (−1.27 mm, *SD* 0.06 mm) and SONIC + A (−1.30 mm, *SD* 0.06 mm), while it was more apical in BUR + A (−3.95 mm, *SD* 0.35 mm).

Mean cross-sectional area was smaller in BUR + A than in SONIC + A (p = .001), and both were smaller with respect to controls (p = .03 and p = .009, respectively) (Figure 5c).

3.6 | Compression tests

No statistically significant difference was found between the mean F_{max} reached in BUR + A (1.51 kN, *SD* 0.17 kN) than in SONIC + A (1.65 kN, *SD* 0.24 kN) (p = .38), and both were not different with respect to controls (1.66 kN, *SD* 0.38 kN) (p = .36 and p = .95, respectively).

Overall, F_{max} was not correlated with volume (p = .92), minimum cross-sectional area (p = .94), position of minimum cross-sectional area (p = .43), or mean cross-sectional area (p = .87) (Figure 6).

4 | DISCUSSION

IP with sonic was found to affect implant structure to a lower extent as compared to burs, in terms of volume reduction, minimum cross-sectional area, and mean cross-sectional area, as observed comparing micro-CT data. The position of the minimum cross-sectional area in relation to the implant platform was similar in controls and SONIC + A groups, whereas it was more apical in BUR + A.

The time taken for IP with sonic was significantly longer than with burs, and the latter treatment resulted in a greater implant weight loss than the former (−11% and −3%, respectively). Surface roughness values Ra and Rz were higher for SONIC versus BUR, while they became similar after polishing with Arkansas in both groups. *SEM* analysis showed a faceted surface after IP with burs,

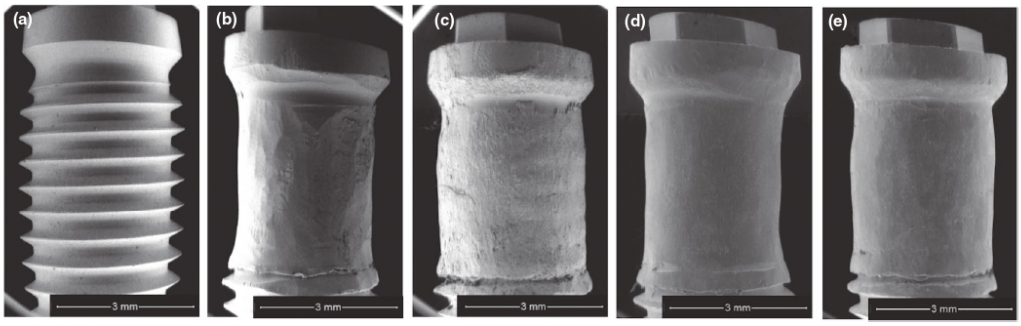

FIGURE 2 *SEM* images of the coronal portion of the implants of: (a) untreated control; (b) BUR; (c) SONIC; (d) BUR + A; and (e) SONIC + A samples

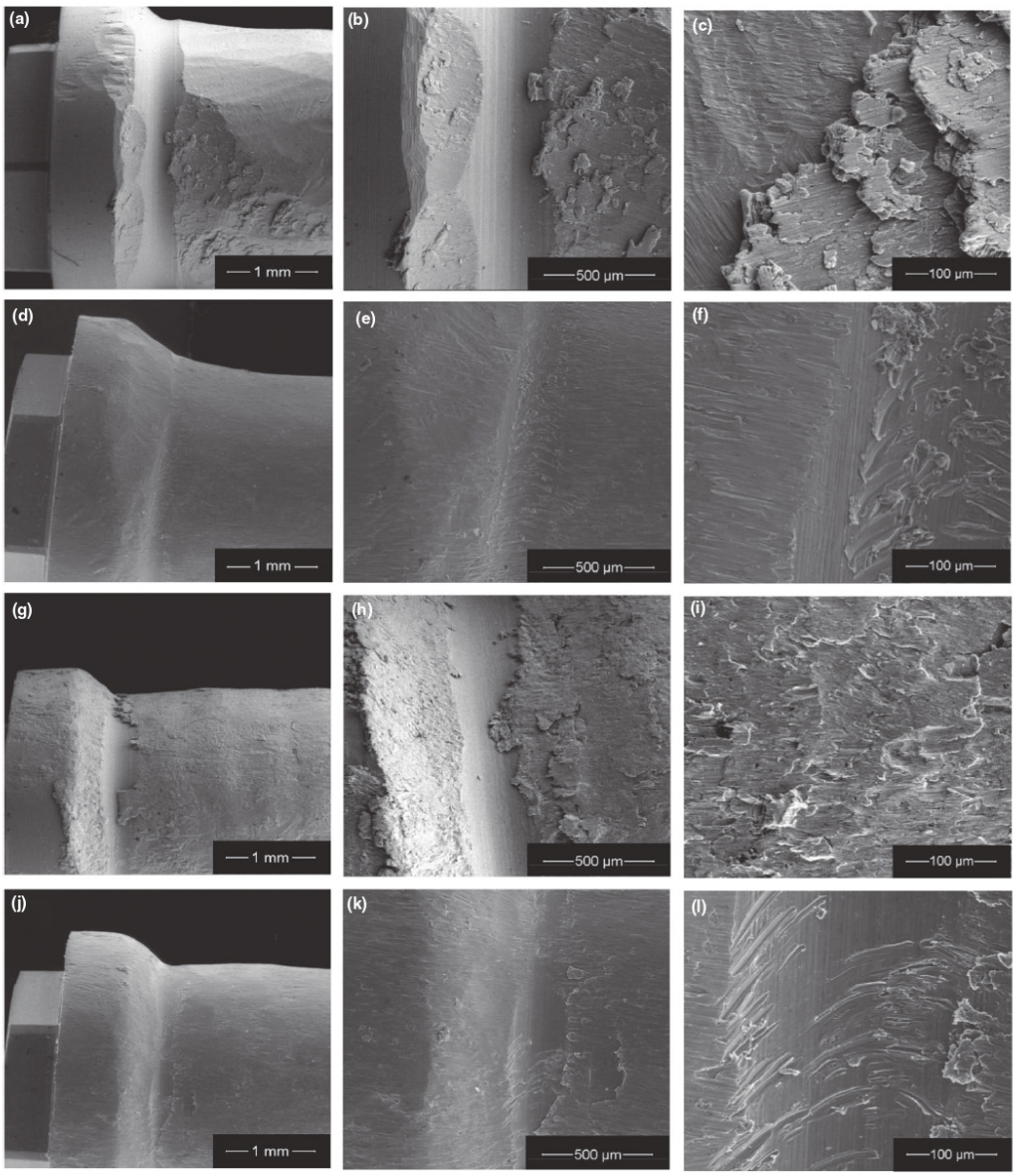

FIGURE 3 *SEM* images at different magnification of: (a,b,c) BUR; (d,e,f) BUR + A; (g,h,i) SONIC; (j,k,l) SONIC + A samples

whereas the sonic left a more homogeneous surface, and differences between the two treatments were minimized after Arkansas, reflecting surface topography findings. Interestingly, SONIC wore implant threads, with a deburring effect. EDS elemental characterization identified Ti as the main surface component in all implants. In addition, C and Al peaks were detected in the sonic and in the Arkansas groups, respectively. These might be attributed to the detachment of diamond particles from the sonic inserts and to the contamination with the Arkansas bur (aluminum oxide stone).

No statistically significant differences were found between BUR + A, SONIC + A, and controls at compression test, even though lower values were registered in BUR + A group, while SONIC + A

TABLE 2 Micro-CT analysis. Data are reported as mean (*SD*)

	Volume [mm^3]	Minimum cross-sectional area [mm^2]	Equivalent diameter of minimum cross-sectional area [mm]	Position of minimum cross-sectional area from platform level [mm]	Mean cross-sectional area [mm^2]
BUR + A	84.51 (2.45)	4.48 (0.62)	2.38 (0.17)	−3.95 (0.35)	5.44 (0.52)
SONIC + A	91.34 (0.81)	5.87 (0.05)	2.73 (0.01)	−1.30 (0.06)	6.77 (0.15)
Controls	94.07 (0.74)	5.77 (0.01)	2.71 (0.01)	−1.27 (0.06)	7.37 (0.05)

Abbreviation: *SD*, standard deviation.

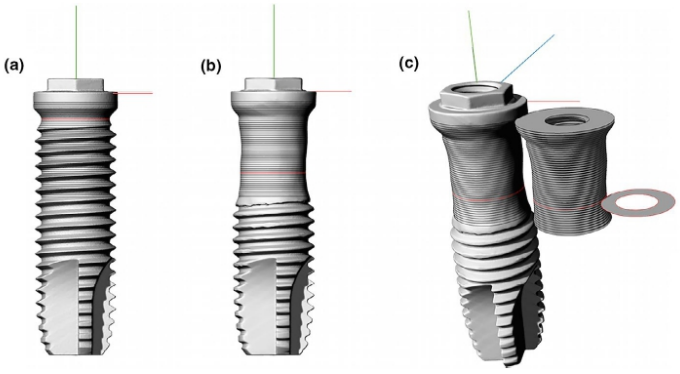

FIGURE 4 Representative micro-CT images of: (a) Control implant; (b) BUR; (c) BUR with highlighted cross-sections and minimum cross-sectional area (right side). Reference coordinate system for STL files is shown with Z (green) axis corresponding to the longitudinal axis of the implant, X (red) and Y (blue) axes parallel to the implant platform plane

and controls were almost equivalent. Moreover, F_{max} was not statistically correlated with any micro-CT parameters.

As regards time associated to BUR, a similar value was reported for the same IP sequence (Costa-Berenguer et al., 2018). However, the longer mean time employed with SONIC + A is also compatible with IP duration reported in other studies. Sahrmann et al. (2019) used a sequence of bud-shaped diamond burs followed by Arkansas and a sequence of conical silicon carbide stone followed by Arkansas and silicone polishers: both procedures recorded a total treatment time of 15 min. Ramel et al. (Ramel et al., 2016) registered an IP duration of 21 ± 4 min with a sequence of diamond burs followed by silicone polishers and 13 ± 2 min with a sequence of diamond burs followed by Arkansas.

Bacterial recolonization after IP is influenced by resulting implant surface roughness. To the best of our knowledge, no in vitro study on IP procedures, included ours, reached values of Ra equal or below the threshold value of 0.2 μm (De Souza Júnior et al., 2016; Ramel et al., 2016; Raoofi et al., 2013; Sahrmann et al., 2019; Tawse-Smith et al., 2016; Toma et al., 2018). The mean Ra value of Osseotite® surface measured in this study was comparable to that found in another study for the same type of surface (Mazor & Cohen, 2003), confirming the repeatability and the precision of surface topography analysis. In the present study, surface roughness values after all IP procedures were higher than those of machined surface and, with the exception of SONIC, lower as compared to Osseotite®. In agreement with other studies (Beheshti Maal et al., 2020; Ramel et al., 2016; Sahrmann et al., 2019), Arkansas finishing smoothed the surface leading to similar roughness between SONIC + A and BUR + A. Raoofi et al., examined the surface roughness obtained by using a sequence of two diamond inserts mounted on a piezosurgery device, obtaining greater values of Ra (1.21 μm, *SD* 0.26) and Rz (3.94 μm, *SD* 1.16) (Raoofi et al., 2013). Ra values measured in SONIC + A were similar to those obtained in another study using a sequence of diamond burs followed by a Greenie silicone polisher (Ra 0.59 μm, *SD* 0.19; Rz 4.35 μm, *SD* 1.37) (Ramel et al., 2016). In the same study, when diamond burs were followed by Arkansas stone, lower values of Ra and Rz (0.39 μm, *SD* 0.13 and 3.19 μm, *SD* 1.17, respectively), were registered. Moreover, the lowest values were obtained with a sequence of diamond burs followed by Brownie and Greenie silicone polishers (Ra 0.32 μm, *SD* 0.14; Rz 2.31 μm, *SD* 0.95). Sahrmann et al. (Sahrmann et al., 2019) reported higher mean values of Ra and Rz (0.76 μm, *SD* 0.14 and 4.12 μm, *SD* 0.72, respectively) compared to that found in SONIC + A and BUR + A, using a sequence of bud-shaped diamond burs followed by Arkansas. However, the authors found that a sequence of conical silicon carbide stone followed by Arkansas, Brownie, and Greenie was able to obtain values of Ra and Rz (0.38 μm, *SD* 0.15 and 1.87 μm, *SD* 0.69, respectively). Therefore, the use of silicone polishers as a surface finishing procedure showed better results than Arkansas in terms of surface roughness (Costa-Berenguer et al., 2018; Sahrmann et al., 2019). Schwarz et al. showed that IP performed with diamond burs followed by Arkansas did not compromise implant surface biocompatibility (Schwarz et al., 2017). The combination of diamond burs and Arkansas stones for IP resulted in a smooth implant surface allowing the adhesion of subepithelial connective tissue in vivo (Schwarz et al., 2011). The use of Arkansas as a polishing procedure

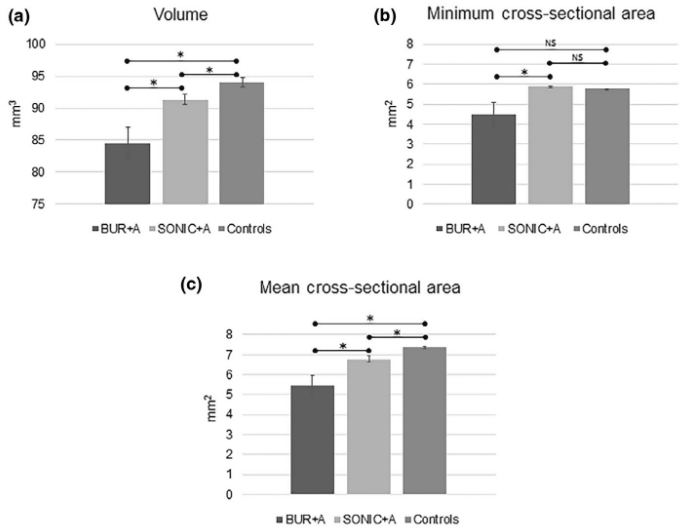

FIGURE 5 Micro-CT analysis: (a) volume; (b) minimum cross-sectional area; and (c) mean cross-sectional area

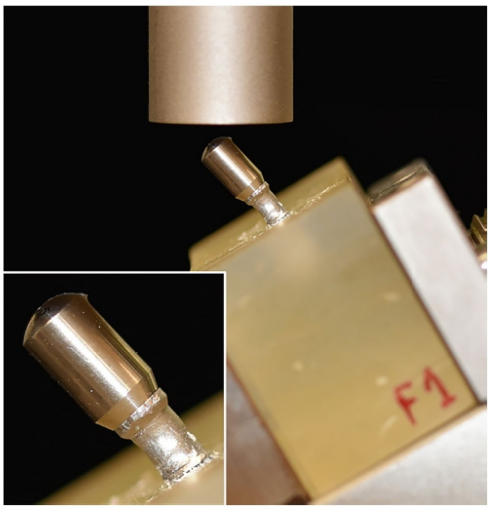

FIGURE 6 Implant deformation is visible after compression test. No statistically significant difference in mean F_{max} was detected between BUR + A, SONIC + A, and controls

is preferable than silicone polishers as it does not cause silicone debris contamination of both implant surface and peri-implant tissues (Beheshti Maal et al., 2020; Costa-Berenguer et al., 2018; Ramel et al., 2016; Tawse-Smith et al., 2016). It has been suggested that the release of Ti micro- and nano-particles from dental implants, such as following IP, can stimulate a number of cytokines, which, in turn, sustain a local inflammatory response (Kumazawa et al., 2002; Noronha Oliveira et al., 2018). In a recent in vitro study using diamond burs for IP on different substrates, the released particles were analyzed in terms of size, ionic product release and effects on human gingival fibroblasts. When cpTi grade IV implants were used, similarly to the present work, the EDS spectra revealed the presence of both Ti and C in the debris. The exposure to these particles did not reduce cell viability in vitro (Barrak et al., 2020). In the present study, metal debris was evident after both procedures. It would be interesting to collect and characterize the particles released following different IP methods, as well as to investigate the biological response in vitro and in vivo.

Micro-CT analysis has been reported for volumetric wear evaluation of meniscus implants (Elsner et al., 2015) or acetabular liners (Teeter et al., 2010). To the best of our knowledge, micro-CT has not yet been applied to assess implant volumetric modifications after in vitro IP. The comparison of implant wear assessed by micro-CT showed significantly higher values in BUR + A when compared to SONIC + A, as confirmed by weight evaluations results. SONIC + A was found to affect the implant structure to a minor extent than BUR + A. Indeed, the weakest section (i.e., minimum cross-sectional area) remained unmodified in SONIC + A with respect to controls, whereas in BUR + A it was reduced and more apically positioned.

In accordance with other studies (Costa-Berenguer et al., 2018; Sahrmann et al., 2019; Tribst et al., 2017), no statistically significant differences were found between implants subjected to different IP procedures with respect to controls in terms of compression resistance value. However, BUR + A showed lower values than SONIC + A and the latter exhibited equivalent values compared to control. It has to be noted that IP is a subtractive procedure, thus a reduction of mechanical strength of the implant should be expected. Chan et al.

reported that 3.75 mm diameter implants presented statistically significant lower maximum bending strengths after IP compared to controls, while this was not true for wider diameters (4.7 mm) (Chan et al., 2013). Gehrke et al. showed that implants fracture strength after IP varied according to the connection design. Morse taper implant connection was proven to be the most resistant followed by external hexagon connection and at last internal hexagon connection (Gehrke et al., 2016). All in vitro studies, including the present one, used implants with a diameter greater than 4 mm with a Morse taper or external hexagon connection (Costa-Berenguer et al., 2018; Sahrmann et al., 2019; Tribst et al., 2017). Therefore, it is reasonable to expect a compressive force reduction after IP in narrower internal hexagon implants. In this case, performing IP with a more conservative method, such as sonic device, could be advantageous.

A final consideration is about the concept of safety applied to the two methods. This in vitro study cannot support the thesis that sonic is safer than burs. However, it was described that burs can frequently cause damage to neighboring teeth (Sahrmann et al., 2019), and it is undeniable that sonic diamond tips could provoke less trauma to soft tissues in case of accidental contact during IP. In restorative dentistry, oscillating diamond instruments are considered particularly useful for tooth preparation in not easily accessible areas as compared to rotary burs (Koubi & Tassery, 2008; Weisrock et al., 2011). Similarly, sonic tips could be of advantage in clinical setting for IP, where the specific configuration of a peri-implant defect may hamper the angulation of a bur, thus compromising IP outcome.

The current cost of sonic tips, higher than that of burs, in relation to their duration could represent a limit in the applicability of the method. Indeed, sonic inserts appeared heavily worn after performing two IP procedures.

A limitation of the present in vitro study could be that IP was performed manually. Indeed, the full control over variables such as pressure, drilling time and area of treatment cannot be achieved. This approach was preferred owing to the similarity to clinical settings.

4.1 | Conclusions

This is the first study describing the use of sonic diamond tips for implantoplasty. Moreover, the use of micro-CT has never been reported so far to investigate the effect of IP on dental implants. IP performed with sonic tips took a longer time to obtain implant surface wear compared to burs; however, it resulted in significantly less material loss. An equivalent surface roughness was achieved after polishing with Arkansas stone. These findings could have a relevant clinical application in case of surgical treatment with implantoplasty of narrow-diameter implants, internal connection implants, or implants located in areas difficult to access by rotary instruments. As it is generally recognized in dentistry that the use of sonic tips is a safer and more conservative method than burs, it would be interesting to develop sonic tips dedicated to IP procedure with adequate shape and specific diamond grain size. Future perspectives may include the study of the biological effect of the debris on cell culture and in preclinical in vivo models, the in vitro assessment of bacteria adhesion and colonization on implant surface after IP, and the evaluation of implant cyclic loading conditions through fatigue tests.

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CONFLICTS OF INTEREST

All the authors declare to have no conflict of interest.

AUTHOR CONTRIBUTION

Stefano Sivolella: Conceptualization (equal); Formal analysis (equal); Writing-original draft (equal); Writing-review & editing (equal). **Giulia Brunello:** Formal analysis (equal); Writing-original draft (equal); Writing-review & editing (equal). **Filippo Michelon:** Investigation (equal); Writing-original draft (equal); Writing-review & editing (equal). **Gianmaria Concheri:** Formal analysis (equal); Writing-review & editing (equal). **Lorenzo Graiff:** Conceptualization (equal); Writing-review & editing (equal). **Roberto Meneghello:** Conceptualization (equal); Formal analysis (equal); Investigation (equal); Writing-original draft (equal); Writing-review & editing (equal).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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APPENDIX A

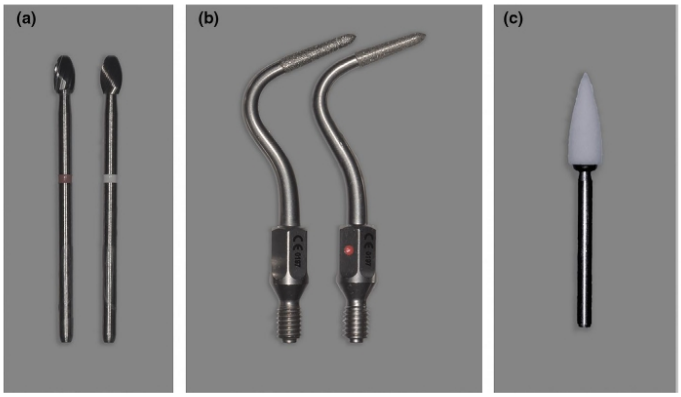

FIGURE A1 (a) Tungsten carbide egg-shaped burs, H379.310.023 (left) and H379UF.310.023 (right) (Komet Dental); (b) torpedo-shaped sonic tips, SF878K.000.018 (left) and SF8878K.000.018 (right), (Komet Dental); and (c) Arkansas bur, FL2 FG 0,244 (Shofu)

TABLE A1 Implant weight, in g

	Weight pre-IP mean (*SD*)	Weight post-IP mean (*SD*)	Weight post-ARKANSAS mean (*SD*)
BUR	0.426 (0.001)	0.386 (0.006)	0.378 (0.009)
SONIC	0.425 (0.003)	0.419 (0.004)	0.411 (0.004)

Abbreviations: IP, implantoplasty; *SD*, standard deviation.

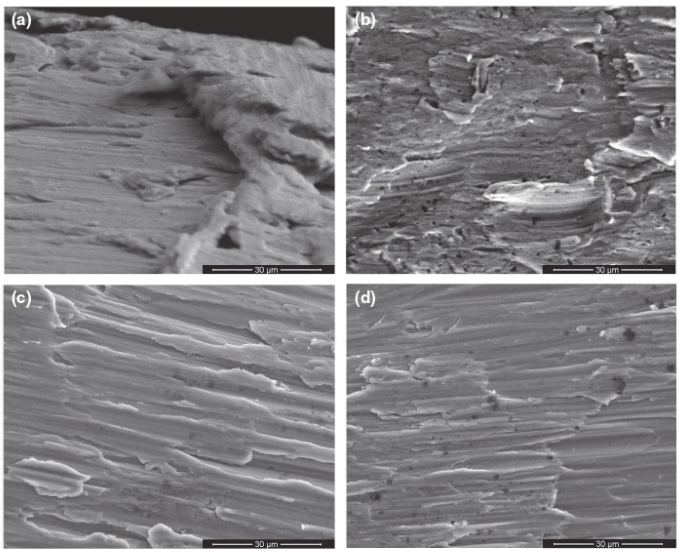

FIGURE A2 *SEM* images at high magnification of: (a) BUR; (b) SONIC; (c) BUR + A; and d) SONIC + A samples

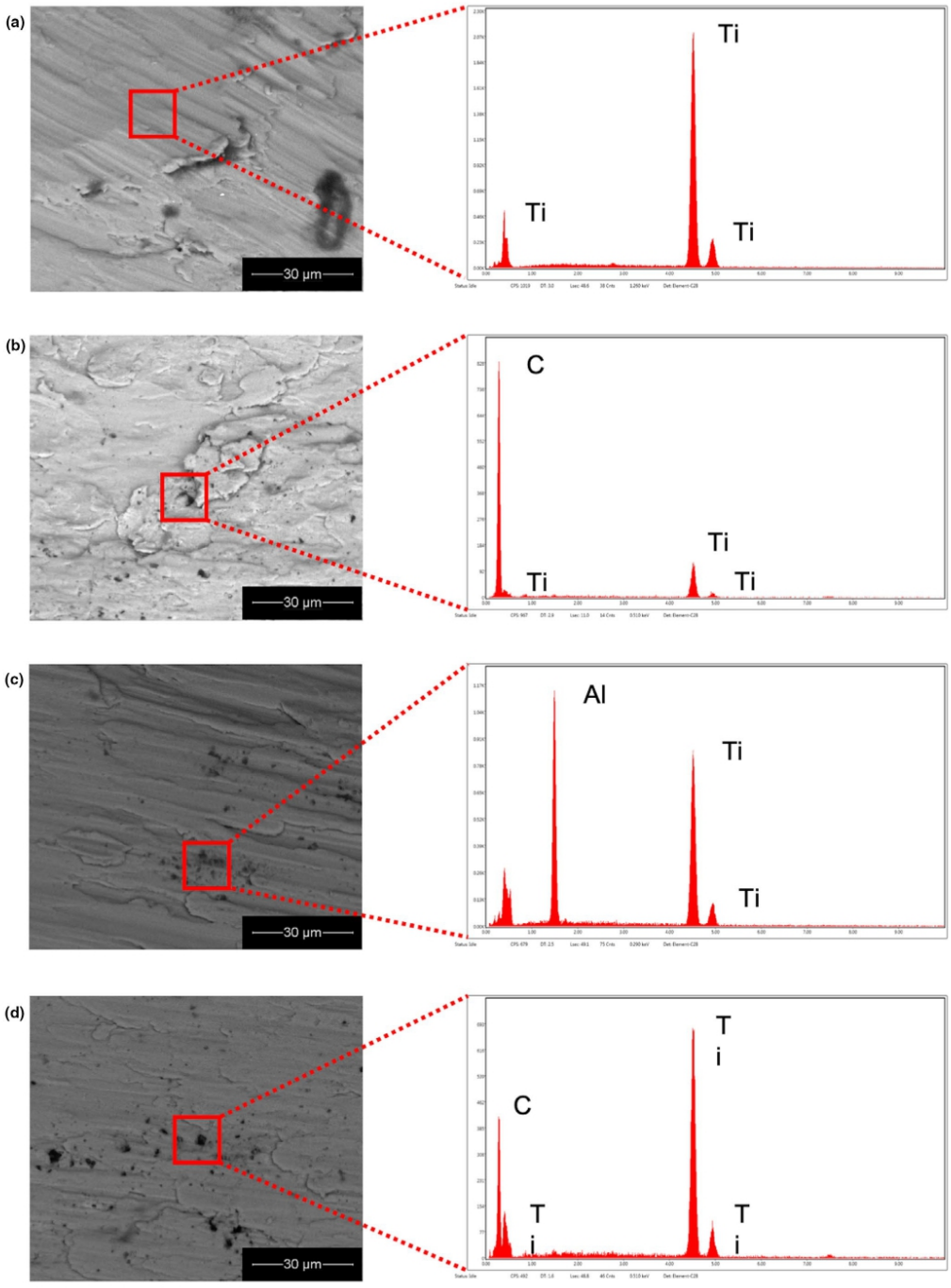

FIGURE A3 *SEM* images and related EDS maps of: (a) BUR; (b) SONIC; (c) BUR + A; and d) SONIC + A samples

2. Original work

antibiotics

MDPI

Article

Efficacy of 0.05% Chlorhexidine and 0.05% Cetylpyridinium Chloride Mouthwash to Eliminate Living Bacteria on In Situ Collected Biofilms: An In Vitro Study

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Abstract: Chlorhexidine (CHX) mouthwashes are frequently used as an adjunctive measure for the treatment of periodontitis and peri-implantitis, as well as in patients on maintenance therapy. However, their prolonged use is associated with several side effects. This study aimed at evaluating if a mouthwash with a reduced concentration of CHX combined with cetylpyridnium chloride (CPC) was as effective as a conventional CHX mouthwash in the reduction in living cells in oral biofilms attached to hydroxyapatite (HA) and micro-rough titanium (Ti) surfaces. Four healthy volunteers wore a customized acrylic appliance containing HA and Ti discs for in situ plaque accumulation. Biofilms were grown on the discs for 24 or 48 h and then randomly exposed for 60 s to: 0.05% CHX + 0.05% CPC, 0.1% CHX (positive control) or sterile saline (negative control). Viability assay and live-dead staining were performed to quantify bacterial viability and to distinguish live and dead cells, respectively. At both time points, contrary to saline, CHX, both alone and in combination with CPC, exhibited high antibacterial properties and induced a significant reduction in biofilm viability. This study demonstrates the potential of mouthwashes containing a low concentration of CHX combined with CPC as effective antibacterial agents for long-term applications with reduced undesired side effects.

Keywords: antiseptic; biofilm; cetylpyridnium chloride; chlorhexidine; mouthrinse; mouthwash; peri-implantitis; periodontitis

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1. Introduction

Periodontal and peri-implant diseases are highly prevalent biofilm-associated inflammatory diseases affecting the supportive structure of teeth or dental implants [1–6]. Gingivitis and mucositis are reversible lesions. Without treatment, however, they can evolve into the more severe and irreversible periodontitis or peri-implantitis, respectively, characterized by connective tissue inflammation and progressive loss of the supporting bone [7,8].

Many studies demonstrated that plaque accumulation plays a crucial role not only in the onset and progression of both pathologies but also in their recurrence [9–12]. Self-performed and professionally administered infection control measures are considered essential in the prevention and treatment of periodontal and peri-implant diseases [13–17], as well as in long-term success after disease resolution [18].

Maintenance becomes particularly important when moderately rough implant surfaces are exposed to the oral cavity. They are widely used owing to the favorable bone

response [19]; however, they also facilitate microbial adhesion, leading to an increased risk of recurrence [20–23].

Beside supportive professional maintenance care programs, adequate self-administered daily home care is recommended. This generally includes the use of a toothbrush, toothpaste and interdental tools, as well as mouthwashes, as adjunctive antiseptic measures to disrupt the biofilms [24,25]. Among these, chlorhexidine (CHX) is most commonly used due to its well-documented antimicrobial activity [26]. However, prolonged CHX usage was also reported to be associated with several drawbacks, such as extrinsic tooth staining, taste disturbance/alteration, burning sensation and loss of efficacy overtime [26–29]. Since these side effects were reported to be dose-dependent [30], low-concentration CHX solutions, combined with other antimicrobials, have been proposed to overcome these drawbacks without losing clinical efficacy [31,32]. Among these adjunctive products, cetylpyridinium chloride (CPC), a cationic surface-active agent belonging to the quaternary ammonium group, is considered to be particularly promising in combination with CHX [33].

Therefore, the goal of the present investigation was to test if a mouthwash with a reduced concentration of CHX (0.05%) and CPC (0.05%) was as suitable as a conventional CHX (0.1%) mouthwash in the reduction in living cells in oral biofilms at hydroxyapatite and micro-rough titanium surfaces.

2. Results

This study was performed in four non-smoking, healthy subjects (two females, two males), aged 25–37 years, with good oral hygiene (plaque index <1). In situ plaque collection was performed at 24 and 48 h. The study adhered to the "Strengthening the Reporting of Observational Studies in Epidemiology" (STROBE) guidelines [34].

2.1. Viability Assay

After 24 h, the highest cell counts per second were recorded for the discs rinsed with NaCl (Figure 1), whereas titanium (Ti) and hydroxyapatite (HA) discs treated with 0.05% CHX + 0.05% CPC (CHX + CPC) and 0.1% CHX (CHX) rinses showed very low counts per second (Figure 1). Significant differences were detected between the NaCl (negative control) and the two other groups (CHX + CPC, CHX), whereas significance failed between the latter (test and positive control) for both surfaces (Table 1).

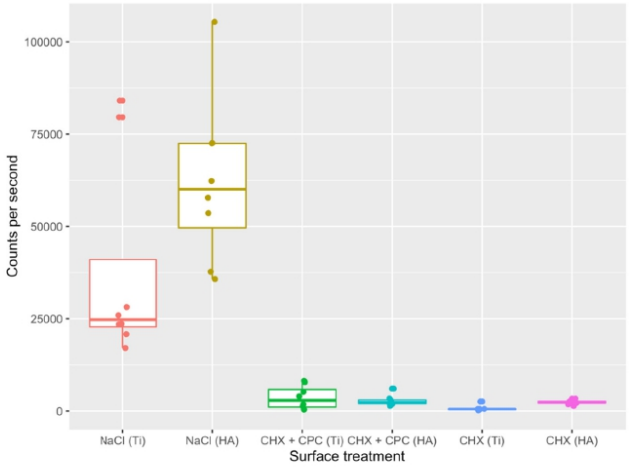

Figure 1. Bacteria viability after 24 h of in situ plaque collection and treatment with NaCl, CHX + CPC or CHX at two types of surfaces (i.e., Ti and HA).

Table 1. A multiple comparison test (Nemenyi post hoc test) was performed to compare the groups after 24 h of in situ plaque collection.

	NaCl (Ti)	NaCl (HA)	CHX + CPC (Ti)	CHX + CPC (HA)	CHX (Ti)
NaCl (HA)	0.98774	-	-	-	-
CHX + CPC (Ti)	0.06271	0.00850 **	-	-	-
CHX + CPC (HA)	0.10432	0.01649 *	0.99996	-	-
CHX (Ti)	0.00014 **	5.9×10^6 ***	0.55798	0.42892	-
CHX (HA)	0.08329	0.01227 *	1.000	1.000	0.48671

The respective *p*-values are provided in the table. Significant values are labeled: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Similarly, after 48 h, the highest cell counts per second were found for the discs rinsed with NaCl (Figure 2), whereas Ti and HA discs treated with CHX + CPC and CHX rinses showed very low counts per second. For Ti surfaces, significant differences were also detected between the NaCl and CHX + CPC and CHX groups, whereas, despite non-overlapping quartile ranges in the boxplot (Figure 2), significance failed between the NaCl (HA) and CHX + CPC (HA) groups (which might be a false negative result owing to the non-parametric test utilized). Additionally, it failed between CHX + CPC and CHX for both investigated surfaces (Table 2).

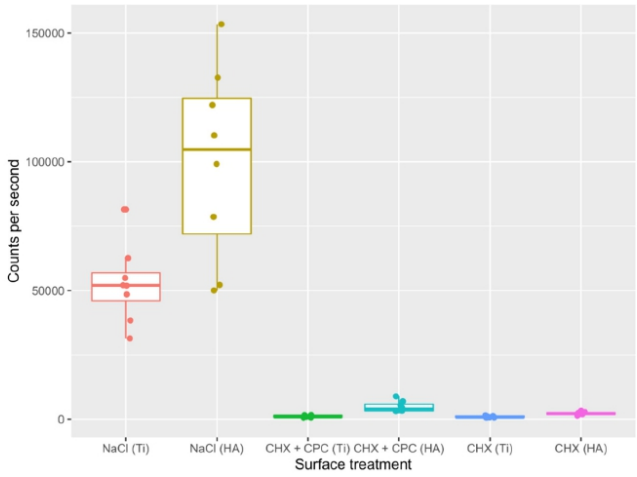

Figure 2. Bacteria viability after 48 h of in situ plaque collection and treatment with NaCl, CHX + CPC or CHX at two types of surfaces (i.e., Ti and HA).

Table 2. A multiple comparison test (Nemenyi post hoc test) was performed to compare the groups after 48 h of in situ plaque collection.

	NaCl (Ti)	NaCl (HA)	CHX + CPC (Ti)	CHX + CPC (HA)	CHX (Ti)
NaCl (HA)	0.96369	-	-	-	-
CHX + CPC (Ti)	0.00100 **	2.4×10^5 ***	-	-	-
CHX + CPC (HA)	0.76306	0.25638	0.08921	-	-
CHX (Ti)	0.00032 ***	6.1×10^6 ***	0.99981	0.04310 *	-
CHX (HA)	0.11882	0.01087 *	0.69282	0.84726	0.51626

The respective *p*-values are provided in the table. Significant values are labeled: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.2. Live-Dead Staining

The live-dead staining procedure allowed distinguishing the living bacteria (labeled in green) from the dead ones (labeled in red). At both 24 and 48 h, no dead bacteria were detected in the NaCl groups regardless of the surface, demonstrating the high viability of the bacterial biofilm (Figures 3 and 4). In contrast, at both time points, almost no living cells could be observed on Ti and HA samples when CHX + CPC and CHX were used. Both solutions showed antimicrobial properties and induced a significant reduction in biofilm viability.

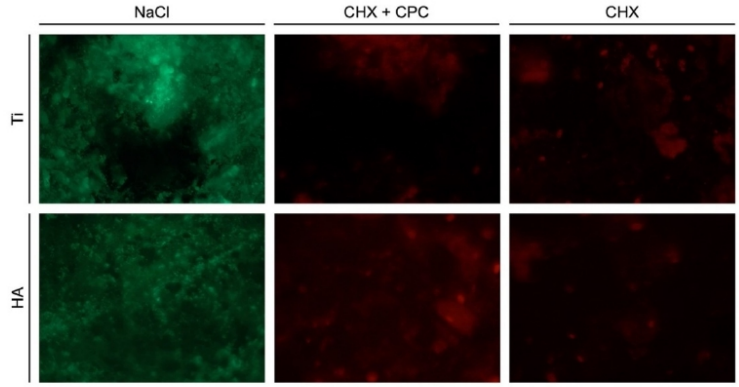

Figure 3. 24-h-old biofilm on Ti and HA surfaces after treatment with NaCl, CHX + CPC or CHX.

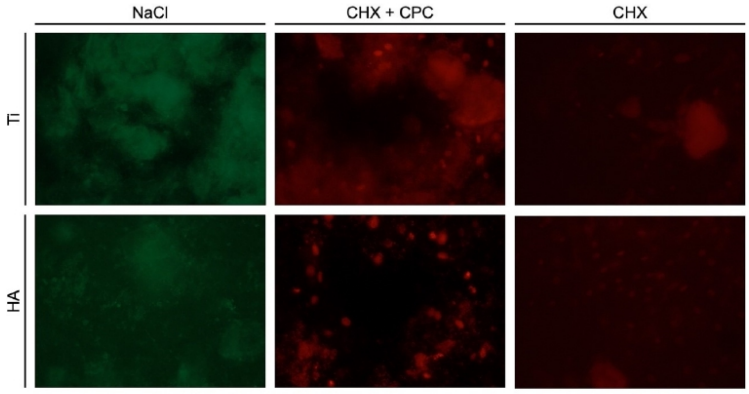

Figure 4. 48-h-old biofilm on Ti and HA surfaces after treatment with NaCl, CHX + CPC or CHX.

3. Discussion

The present study aimed at evaluating the efficacy, in terms of the reduction in vital bacteria, of a mouthwash containing a low concentration of chlorhexidine in combination with cetylpyridinium chloride (CHX + CPC) as compared to the widely used chlorhexidine 0.1% (CHX) mouthwash. In order to mimic the exposure of tooth and implant surfaces to an oral biofilm, discs made of hydroxyapatite (HA) and of a commonly used titanium implant surface (Promote®, CAMLOG Biotechnologies AG, Basel, Switzerland) (Ti) were utilized for in situ plaque collection.

At 24 and 48 h, significant differences were recorded between the sterile saline group (NaCl) and the other two groups, i.e., CHX + CPC and CHX, when applied on Ti surfaces. Interestingly, and despite the non-overlapping interquartile ranges, no significant difference was identified between NaCl and CHX + CPC on HA surfaces. However, as no differences were found between the CHX + CPC and CHX groups in all experimental conditions, the present results indicate that both CHX groups demonstrated comparable efficacy.

Cetylpyridinium chloride is a quaternary ammonium compound, included in the group of cationic surface-active agents, and originally, it demonstrated only moderate efficacy [35]. However, when combined with chlorhexidine, a synergistic effect is assumed, increasing the overall antimicrobial activity [36].

Self-administered antiseptic mouthwashes, as an adjunctive measure to mechanical debridement for patients in supportive periodontal care, were frequently reported to be effective in reducing plaque accumulation, in decreasing the proportion of bacteria from the red and orange spectrum and in the reduction in probing depths [37,38]. Few studies investigated the efficacy of the combination of cetylpyridinium chloride and chlorhexidine and demonstrated a reduction in plaque levels and bacterial counts [32], as well as in bleeding on probing (BOP) scores [31]. A double-blind randomized controlled trial (RCT) compared the adjunctive use of 0.05% CPC and 0.05% CHX (with and without alcohol) with 0.2% CHX and found both effective in improving plaque and gingivitis indices [39].

For peri-implant mucositis, the beneficial effect of an antiseptic mouthwash as an adjunctive measure to mechanical debridement remains controversial [40,41]. Two studies (reporting on the same sample of patients) investigated the long-term efficacy, i.e., up to 12 months of follow-up, of 0.03% CHX and 0.05% CPC as an adjunct to professionally and patient-administered mechanical plaque removal in the treatment of peri-implant mucositis. The tested mouthwash resulted in a significant higher reduction in buccal BOP values compared to the placebo mouthwash [42,43]. In both studies, a placebo mouthwash was used as a control in the treatment of peri-implant mucositis, while no comparison was performed with chlorhexidine at higher concentrations.

Therefore, limited evidence exists regarding the efficacy of antiseptic mouthwashes as an adjunctive measure for patients with peri-implant tissue inflammation. The present study demonstrated comparable antimicrobial properties on Ti and Ha surfaces for the combination of 0.05% CPC and 0.05% CHX. In situ plaque collection was selected, as it is considered a useful tool mimicking the normal biofilm development, which is characterized by high complexity and by the presence of numerous bacterial strains [44,45]. By contrast, in vitro cultivation of bacterial biofilms is not likely to mirror the architecture and the composition of the in vivo biofilms. However, specific pathogens can be selected for cultivation that may not be contained in biofilms retrieved from healthy volunteers [44,46]. In the present study, four periodontally healthy volunteers wore acrylic appliances with Ti and HA discs to build up supragingival plaque. Nonetheless, a shortcoming of this approach might consist in the selection of the participants, whose microbiota could differ from that in patients with a history of periodontal or peri-implant disease [47], who represent the target of prolonged use of tested mouthwashes. A larger and more representative pool of participants could be used in future investigations.

Further limitations include the absence of biofilm characterization and of cytocompatibility tests. Regarding the latter, the oral cavity contains different cells, including fibroblasts and epithelial cells. As the mouthwashes are meant to be in contact with the oral mucosa, beside their antimicrobial properties, cell compatibility should also be investigated [48,49].

Finally, corrosion seems to affect dental implants' biocompatibility, leading to their long-term failure [50]. Although both 0.12% CHX gluconate and 0.5% solutions did not alter the corrosive behavior of sandblasted, acid-etched Ti surfaces in vitro [51], it would be interesting to investigate the effect of the mouthwashes utilized in the present study on commonly used dental implant surfaces.

Furthermore, in relation to the ongoing COVID-19 pandemic, CHX- and CPC-based pre-procedural mouthwashes might also be effective in reducing the risk of SARS-CoV-2 transmission in dental settings, as recently suggested [52,53].

In summary, the present study is in line with previous investigations demonstrating the efficacy of the 0.05% CHX + 0.05% CPC formulation, which permits the use of lower concentrations of CHX while maintaining high antibacterial properties.

4. Materials and Methods

4.1. Study Population

Four healthy subjects (age ≥ 18 years, non-smokers, good oral hygiene and health, plaque index < 1, no antibiotic therapy within the last 6 months, absence of periodontal diseases as per Papapanou et al. [54]) were included for the collection of the biofilm. All volunteers signed a written consent form before participating in the present study. The study protocol was approved by the Ethics Committee of the University of Düsseldorf (Protocol no. 5797R). The study was conducted following the recognized standards of the Declaration of Helsinki and the European Medicines Agency Guidelines for Good Clinical Practice. The present study was also performed and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines [34].

4.2. In Vivo Biofilm Formation

A customized acrylic appliance for the upper jaw was produced for each subject, containing a range of 27 to 34 discs (2 mm in thickness and 5 mm in diameter) of two different materials, i.e., hydroxyapatite (HA) provided by Dentaid® GmbH (Barcelona, Spain) and titanium (Ti) with moderately rough Promote® surface (CAMLOG Biotechnologies AG, Basel, Switzerland). The subjects were randomly allocated to wear the appliance for either 24 or 48 h to achieve in situ plaque collection. The subjects were permitted to take off the appliance during eating and to perform mechanical tooth brushing without toothpaste or any other chemical adjuncts. The customized appliances were fabricated as described in John et al. [55]. Briefly, the discs were glued in impression to the palatal side of the appliance with a cyanoacrylate glue (Loctide® 496, Henkel AG & Co. KGaA, Düsseldorf, Germany), leaving a 1-mm distance between the palatal mucosa and the disc surface exposed to the oral cavity.

After plaque accumulation for 24 or 48 h, the disks were collected and gently rinsed with sterile water to remove macroscopic food debris and randomly assigned to the following treatment groups: test group 0.05% CPC + 0.05% CHX (PERIO-AID® Active Control, Dentaid® GmbH, Barcelona, Spain) (CPC + CHX), positive control 0.1% CHX (Chlorhexamed® Fluid 0.1%, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG, Bühl, Germany) and negative control (sterile saline). The application time of the mouthwashes was 60 s.

Viability assays were used to quantify bacterial viability. Additionally, live-dead staining was performed for descriptive purposes.

4.3. Viability Assay

A total of 96 discs, 8 per group at both time points, were used for the assessment of bacterial viability. Immediately after treatment with the mouthwashes, the discs were transferred to 96-well plates. The bacterial viability was measured using the BacTiter-Glo® luminescent viability assay kit (Promega, Madison, WI, USA), following the instructions of the manufacturer. This test is based on the luciferase-catalyzed reaction of luciferin and adenosine triphosphate (ATP) and, hence, quantifies the ATP present, which indicates the presence of metabolically active cells.

Briefly, 100 µL of BacTiter-Glo® reagent was added to the wells and incubated in darkness at room temperature. The luminescent signal was then recorded using a luminometer (Victor 2030, PerkinElmer, Rodgau, Germany).

4.4. Live-Dead Staining

Live-dead staining was performed on 3 samples per group and time point. For fluorescent sample staining, LIVE/DEADTM BacLight^{TM} Bacterial Viability Kit (Thermo Fisher Scientific, Wesel, Germany) was utilized, and photographs were then taken (ColourView III, Olympus Europa GmbH, Hamburg, Germany) using a stereomicroscope (SZ61, Olympus Europa GmbH, Hamburg, Germany).

4.5. Statistical Analysis

Statistical evaluation was conducted using the software R [56]. For each time point, surface and mouthwash, boxplots were created for descriptive purposes. The Kruskal-Wallis test and the post hoc multiple comparison Nemenyi test with the Tukey method for *p*-value adjustment were used to assess statistical differences in bacterial viability among the three treatment groups (applied at two surfaces) per time point. The results were found significant at $p < 0.05$.

5. Conclusions

In conclusion, within the limitations of the present study, both 0.05% CHX + 0.05% CPC and 0.1% CHX solutions exhibited comparable antibacterial properties when used to rinse hydroxyapatite and titanium surfaces. Due to the reduced concentration of CHX, the combination of CPC and CHX might be beneficial for long-term application.

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Institutional Review Board Statement: The study protocol was approved by the Ethics Committee of the University of Düsseldorf (Protocol no. 5797R). The study was conducted following the recognized standards of the Declaration of Helsinki and the European Medicines Agency Guidelines for Good Clinical Practice.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data will be provided upon reasonable request.

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3. Original work

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The Effects of Three Chlorhexidine-Based Mouthwashes on Human Osteoblast-Like SaOS-2 Cells. An In Vitro Study

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Abstract: Several decontamination methods for removing biofilm from implant surfaces during surgical peri-implantitis treatment have been reported, including the intraoperative usage of chlorhexidine (CHX)-based antiseptics. There is a lack of information on possible adverse effects on bone healing. The study aimed to examine the impact of three CHX-based mouthwashes on osteoblast-like cells (SaOS-2) in vitro. Cells were cultured for three days in 96-well binding plates. Each well was randomly treated for either 30, 60 or 120 s with 0.05% CHX combined with 0.05% cetylpyridinium chloride (CPC), 0.1% CHX, 0.2% CHX or sterile saline (NaCl) as control. Cell viability, cytotoxicity and apoptosis were assessed at day 0, 3 and 6. Cell viability resulted in being higher in the control group at all time points. At day 0, the CHX 0.2 group showed significantly higher cytotoxicity values compared to CHX 0.1 (30 s), CHX + CPC (30 s, 60 s and 120 s) and control (60 s and 120 s), while no significant differences were identified between CHX + CPC and both CHX 0.1 and NaCl groups. All test mouthwashes were found to induce apoptosis to a lower extent compared to control. Results indicate that 0.2% CHX presented the highest cytotoxic effect. Therefore, its intraoperative use should be carefully considered.

Keywords: antiseptic; bone; cetylpyridinium chloride; chlorhexidine; mouthrinse; peri-implantitis; periodontitis

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1. Introduction

Peri-implantitis is a multifactorial bacteria-induced pathology affecting the peri-implant tissues, leading to a progressive reduction of the supporting bone and, subsequently, to implant loss if left untreated [1,2].

While a non-surgical mechanical debridement might be resolutive in the case of peri-implant mucositis, it seems to have limited efficacy for the management of peri-implantitis [3,4]. Although the non-surgical approach represents a fundamental step in the initial treatment of peri-implantitis, in cases of recurrence of bleeding and suppuration, it has to be followed by surgical therapy, which allows a better access for an effective removal of the biofilm from the contaminated implant surfaces [5]. To this aim, several mechanical and chemical techniques have been proposed; however, no particular decontamination protocol has been demonstrated to be superior [5–7].

Mouthwashes can be used as adjunctive measures to the mechanical elimination of bacteria through surgical debridement [8]. Among these, chlorhexidine (CHX) is one of the most commonly used products due to its high antibacterial properties [5,9]. However,

its beneficial effect is controversial. In a randomized controlled clinical trial on the surgical treatment of advanced peri-implantitis, a 0.2% solution of chlorhexidine digluconate did not exhibit any beneficial effect over the mechanical implant surface decontamination alone at both 1- and 3-year follow-up [10,11]. This is consistent with previous findings in animal models [12]. Furthermore, socket rinsing with CHX has also been proposed, but its effect is still controversial, as some authors reported impairment of wound healing while others reported a reduced rate of alveolitis [13,14].

Several concerns have been raised regarding the potential tissue toxicity of these agents. Numerous studies have investigated the cytotoxicity of CHX on different cells, including fibroblasts, osteoblasts, myoblasts and epithelial cells [15–24]. In particular, for these clinical applications, CHX-based solutions would be in direct contact with the bone and the connective tissues without the protective barrier of the intact epithelium, thus increasing the risk of cytotoxicity [16,21]. Regarding osteoblasts, 0.1% CHX has been reported to rapidly induce morphological changes and cell damage in human osteoblasts already after an incubation time of one minute [25]. In John et al. [20], 0.2% CHX was found to be cytotoxic for SaOS-2 cells. In a study investigating the effect of CHX on the same cell line, cell viability was reduced in dose- and time-dependent manners [26]. A dose-dependent CHX cytotoxicity was also observed in other in vitro studies [15,17,23].

Furthermore, CHX-induced perioperative hypersensitivity has been extensively reported in the literature [27]. Although severe reactions are rarely observed in relation to mouthwashes, rinsing with an open flap might increase the risk of their occurrence.

To reduce the CHX-related side effects, shorter exposure time and/or lower concentration of CHX alone or in combination with additional compounds have been recommended. The combination CHX and cetylpyridinium chloride (CPC) has been demonstrated to be effective when used as an adjunct to oral hygiene for patients in supportive periodontal care [28–30], as well as in cases of peri-implant mucositis [31,32]. A 0.12% CHX + 0.05% CPC solution was found to reduce bacterial load to a greater extent than mechanical debridement alone in respective peri-implantitis treatment [33] and exhibited similar clinical, radiographic and microbiological outcomes as compared to an alcohol containing 0.2% CHX [34]. Nevertheless, whether its additional use translates into enhanced clinical outcomes remains to be clarified.

In a recent study by our team [35], contrary to saline, two commercially available CHX-based mouthwashes (i.e., 0.05% CHX + 0.05% CPC and 0.1% CHX) were found to be effective in the reduction of living bacteria in oral biofilms attached to micro-rough titanium surfaces. Following a 60 s exposure to the mouthwashes, no significant difference was found between the two groups in bacteria viability after 24 as well as 48 h of in situ plaque collection.

Taking into consideration the remarkable antibacterial properties exhibited by a 0.05% CHX + 0.05% CPC mouthwash and the well-documented cytotoxicity associated with antiseptics containing CHX at higher concertation, the relevant clinical question arose of whether it can be safely used as adjunctive in the surgical treatment of peri-implantitis.

Although numerous studies have investigated the effect of different concentrations of CHX on osteoblasts, to the best of our knowledge, the effects of a low-concentration CHX solution containing CPC on osteoblasts had not been explored yet. Therefore, the aim of this study was to evaluate in vitro the effects of three commercially available mouthwashes containing CHX at different dilutions, alone or in combination with CPC, on osteoblast-like cells by examining cell viability, cytotoxicity and apoptosis.

2. Results

Results on cell viability, cytotoxicity and apoptosis are reported below. No cell culture was lost due to microbial contamination.

2.1. Cell Viability

The highest cell viability values were predictably detected in the control group (NaCl) at all time points, as shown in Figure 1. Unexpectedly and not in accordance with the descriptive analysis illustrated in the boxplot, no significant difference was identified between CHX 0.2 and NaCl groups at day 0 (30 s, 60 s and 120 s) as well as at day 3 (120 s) (Table 1). The *p*-values presented in Table 1 are Bonferroni-corrected. The uncorrected *p*-values were <0.05 in all these cases but one (30 s day 0: $p = 0.083$).

Within all test groups, application time did not affect cell viability, except for CHX 0.1 at day 0. Indeed, significant differences were observed between 30s and the longer application times (i.e., 30 s vs. 60 s; 30 s vs. 120 s).

Only at day 0, statistically significant differences were found between the test groups. In detail, CHX 0.2 presented higher values compared to CHX 0.1 and CHX + CPC after an application time of 30 s and 120 s, respectively.

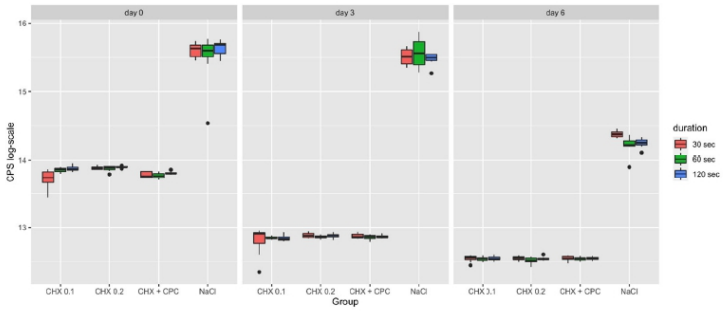

Figure 1. Boxplot representing the cell viability of SaOS-2 cells following the different treatment procedures (i.e., CHX 0.1, CHX 0.2, CHX + CPC and NaCl for 30, 60 and 120 s) at day 0, 3 and 6. Data are expressed in counts per second (CPS).

Table 1. Cell viability. A multiple Kruskal–Wallis test was performed to compare the groups at each time point (i.e., at day 0, 3 and 6), and in the case of significance, a post hoc multiple comparison test with Bonferroni *p*-value adjustment was performed. The adjusted *p*-values from post hoc test are reported and labeled as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Grouping Variable	Comparator 1	Comparator 2	*p*-Value (Day 0)	*p*-Value (Day 3)	*p*-Value (Day 6)
CHX 0.1	30 s	60 s	0.040 *	-	-
	30 s	120 s	0.005 **	-	-
	60 s	120 s	1.000	-	-
CHX 0.2	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
CHX + CPC	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
NaCl	30 s	60 s	-	-	0.003 **
	30 s	120 s	-	-	1.000
	60 s	120 s	-	-	0.008 **
30 s	CHX 0.1	CHX 0.2	0.033 *	1.000	1.000
	CHX 0.1	CHX + CPC	1.000	1.000	1.000
	CHX 0.1	NaCl	0.000 ***	0.006 **	0.007 **

	CHX 0.2	CHX + CPC	0.141	1.000	1.000
	CHX 0.2	NaCl	0.499	0.007 **	0.004 **
	CHX + CPC	NaCl	0.000 ***	0.001 **	0.002 **
60 s	CHX 0.1	CHX 0.2	1.000	1.000	1.000
	CHX 0.1	CHX + CPC	0.310	1.000	1.000
	CHX 0.1	NaCl	0.017 *	0.001 **	0.007 **
	CHX 0.2	CHX + CPC	0.054	1.000	1.000
	CHX 0.2	NaCl	0.123	0.008 **	0.000 ***
	CHX + CPC	NaCl	0.000 ***	0.009 **	0.016 *
120 s	CHX 0.1	CHX 0.2	1.000	0.733	1.000
	CHX 0.1	CHX + CPC	0.274	1.000	1.000
	CHX 0.1	NaCl	0.014 *	0.000 ***	0.003 **
	CHX 0.2	CHX + CPC	0.024 *	1.000	1.000
	CHX 0.2	NaCl	0.185	0.050	0.004 **
	CHX + CPC	NaCl	0.000 ***	0.004 **	0.006 **

2.2. Cytotoxicity

At day 0, the highest cytotoxicity was detected in the CHX 0.2 group, which presented significantly higher values compared to CHX 0.1 (30 s), CHX + CPC (30 s, 60 s and 120 s) and control (60 s and 120 s). Lower cytotoxicity was exhibited by the CHX 0.1 as compared to NaCl after an application time of 30 s, while the opposite was observed after 120 s ($p < 0.05$ and $p < 0.01$, respectively). For all the exposure times, no significant differences were found between CHX + CPC and CHX 0.1, as well as between CHX + CPC and the control. Moreover, at day 0, the application time was not found to be determinant within the CHX 0.2 group. An increased cytotoxicity dependent on the application time was observed in both CHX 0.1 (60 s > 30 s and 120 s > 30 s) and CHX + CPC (120 s > 30 s). By contrast, within the NaCl, results are inverted, with longer application times associated to a lower cytotoxicity compared to 30 s exposure.

As evidenced in the graph (Figure 2), at day 3 and day 6, a similar situation was observed, with the highest values recorded in the control group compared to the others. Contrary to day 0, application time was found not to be relevant in the majority of cases. Likewise, for cell viability test, in contrast with what was reported in the boxplot, no significant difference was identified between CHX 0.2 and NaCl at day 3 (30 s and 60 s) and between CHX 0.1 and NaCl at day 6 (30 s) (Table 2). The *p*-values presented in Table 2 are Bonferroni-corrected. The respected uncorrected *p*-values were <0.05 in all three cases.

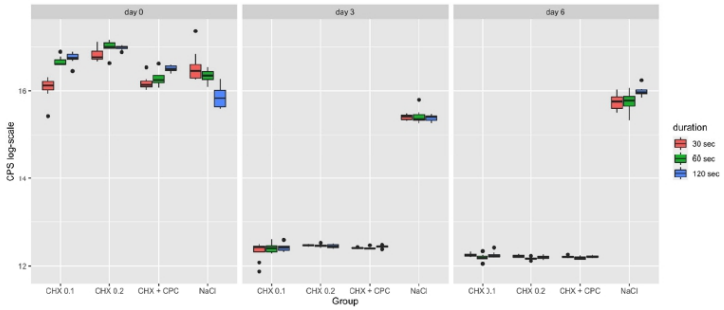

Figure 2. Boxplot representing the cytotoxicity on SaOS-2 cells following the different treatment procedures (i.e., CHX 0.1, CHX 0.2, CHX + CPC and NaCl for 30, 60 and 120 s) at day 0, 3 and 6. Data are expressed in counts per second (CPS).

Table 2. Cytotoxicity. A multiple Kruskal–Wallis test was performed to compare the groups at each time point (i.e., at day 0, 3 and 6), and in the case of significance, a post hoc multiple comparison test with Bonferroni *p*-value adjustment was performed. The adjusted *p*-values from post hoc test are reported and labeled as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Grouping Variable	Comparator 1	Comparator 2	*p*-Value (Day 0)	*p*-Value (Day 3)	*p*-Value (Day 6)
CHX 0.1	30 s	60 s	0.011 *	-	-
	30 s	120 s	0.000 ***	-	-
	60 s	120 s	0.967	-	-
CHX 0.2	30 s	60 s	-	-	0.250
	30 s	120 s	-	-	0.014 *
	60 s	120 s	-	-	0.819
CHX + CPC	30 s	60 s	0.916	0.231	-
	30 s	120 s	0.004 **	0.030 *	-
	60 s	120 s	0.085	1.000	-
NaCl	30 s	60 s	0.014 *	-	1.000
	30 s	120 s	0.001 **	-	0.027 *
	60 s	120 s	1.000	-	0.071
30 s	CHX 0.1	CHX 0.2	0.000 ***	0.695	1.000
	CHX 0.1	CHX + CPC	1.000	1.000	0.733
	CHX 0.1	NaCl	0.043 *	0.002 **	0.068
	CHX 0.2	CHX + CPC	0.002 **	0.073	1.000
	CHX 0.2	NaCl	1.000	0.241	0.002 **
	CHX + CPC	NaCl	0.123	0.000 ***	0.000 ***
60 s	CHX 0.1	CHX 0.2	0.559	0.695	1.000
	CHX 0.1	CHX + CPC	0.054	1.000	1.000
	CHX 0.1	NaCl	0.141	0.001 **	0.009 **
	CHX 0.2	CHX + CPC	0.000 ***	0.472	1.000
	CHX 0.2	NaCl	0.000 ***	0.131	0.001 **
	CHX + CPC	NaCl	1.000	0.000 ***	0.006 **
120 s	CHX 0.1	CHX 0.2	0.420	1.000	1.000
	CHX 0.1	CHX + CPC	0.947	1.000	1.000
	CHX 0.1	NaCl	0.006 **	0.001 **	0.033 *
	CHX 0.2	CHX + CPC	0.008 **	1.000	1.000
	CHX 0.2	NaCl	0.000 ***	0.017 *	0.001 **
	CHX + CPC	NaCl	0.373	0.005 **	0.002 **

2.3. Apoptosis

At all time points, the highest apoptotic levels were registered in the NaCl control group (Figure 3). As reported in Table 3, within each treatment and control group, at day 0 the application time was not found to significantly influence the apoptotic effect on SaOS-2 cells. At day 3, significant differences in apoptosis were observed between the 30 s and 120 s application times both in the NaCl and CHX + CPC. On the other hand, at day 6, significant differences were registered within all groups but one (i.e., CHX 0.2).

Unexpectedly and not in accordance with what was reported in the boxplot (Figure 3), there was no statistically significant difference between NaCl and CHX + CPC groups at day 3 (120 s) and at day 6 (30 s). The *p*-values presented in Table 3 are Bonferroni-corrected. The uncorrected *p*-values were <0.05 in both cases.

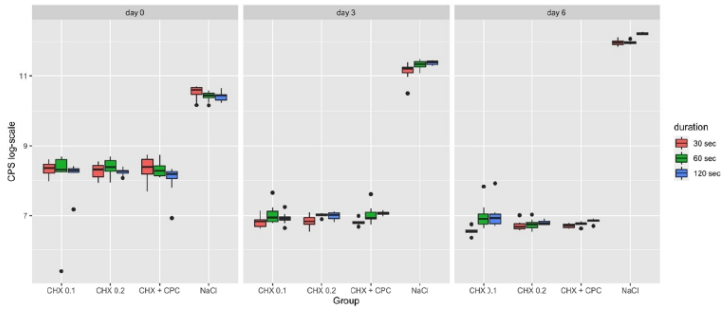

Figure 3. Boxplot representing the apoptosis of SaOS-2 cells following the different treatment procedures (i.e., CHX 0.1, CHX 0.2, CHX + CPC and NaCl for 30, 60 and 120 s) at day 0, 3 and 6. Data are expressed in counts per second (CPS).

Table 3. Apoptosis. A multiple Kruskal–Wallis test was performed to compare the groups at each time point (i.e., at day 0, 3 and 6), and in the case of significance, a post hoc multiple comparison test with Bonferroni *p*-value adjustment was performed. The adjusted *p*-values from post hoc test are reported and labeled as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Grouping Variable	Comparator 1	Comparator 2	*p*-Value (Day 0)	*p*-Value (Day 3)	*p*-Value (Day 6)
CHX 0.1	30 s	60 s	-	-	0.005 **
	30 s	120 s	-	-	0.003 **
	60 s	120 s	-	-	1.000
CHX 0.2	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
CHX + CPC	30 s	60 s	-	0.078	0.915
	30 s	120 s	-	0.002 **	0.003 **
	60 s	120 s	-	0.730	0.074
NaCl	30 s	60 s	-	0.121	1.000
	30 s	120 s	-	0.014 *	0.004 **
	60 s	120 s	-	1.000	0.009 **
30 s	CHX 0.1	CHX 0.2	1.000	1.000	0.320
	CHX 0.1	CHX + CPC	1.000	1.000	0.173
	CHX 0.1	NaCl	0.004 **	0.004 **	0.000 ***
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.001 **	0.006 **	0.026 *
	CHX + CPC	NaCl	0.010*	0.002 **	0.056
60 s	CHX 0.1	CHX 0.2	1.000	1.000	1.000
	CHX 0.1	CHX + CPC	1.000	1.000	1.000
	CHX 0.1	NaCl	0.006 **	0.002 **	0.048 *
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.006 **	0.011 *	0.001 **
	CHX + CPC	NaCl	0.002 **	0.002 **	0.002 **
120 s	CHX 0.1	CHX 0.2	1.000	1.000	0.878
	CHX 0.1	CHX + CPC	1.000	0.472	1.000
	CHX 0.1	NaCl	0.020 *	0.000 ***	0.036 *
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.006 **	0.004 **	0.000 ***
	CHX + CPC	NaCl	0.000 ***	0.068	0.006 **

3. Discussion

The purpose of this study was to determine the impact of three commercially available mouthwashes containing chlorhexidine (CHX) at different concentrations, alone or in combination with cetylpyridinium chloride (CPC), on osteoblast-like cells (SaOS-2) cultured for 2 h, 3 days and 6 days after different exposure times to the mouthwashes tested in this study (i.e., 30 s, 60 s and 120 s).

Among the tested mouthwashes, the highest cell viability values were predictably recorded in the NaCl (control) group at all three time points, and for all application times. Except for day 0, in which CHX 0.2 showed higher values than CHX 0.1 at 30 s application time as well as higher values than CHX + CPC at an application time of 120 s, cell viability was comparable among the tested mouthwashes. The application time did not reveal any effect on cell viability within the test groups except for CHX 0.1 at day 0.

Besides cell viability, the triplex assay utilized in the present study allowed for exploring the SaOS-2 death mechanism induced by the different treatment procedures. Contrary to apoptosis, which is characterized by cell membrane integrity, its disruption and the subsequent release of the cytoplasmic contents into the surrounding tissue occur in case of necrosis [36,37].

Hence, in virtue of the different morphological features of these two cellular death mechanisms, it was possible to assess the cytotoxicity by means of a fluorogenic proteolytic biomarker that is released from cells that have lost their membrane integrity. The exposure to CHX 0.2 resulted in significantly higher cytotoxicity levels at day 0 compared to CHX 0.1 (30s), CHX + CPC (30 s, 60 s and 120 s) and NaCl (60 s and 120 s). No significant differences were found between CHX + CPC and both CHX 0.1 and NaCl for all the application times. Interestingly, CHX + CPC as well as CHX 0.1 exhibited a time-dependent cytotoxicity on SaOS-2, whereas an inverse correlation between application time and cytotoxic effect was observed in the NaCl group at day 0. This finding can hardly be explained, as longer rinsing procedures were expected to be associated with higher cellular stress. Nevertheless, as emerged from cell viability assay, well recovery of the cells was evidenced in the control group at day 3 and 6. By contrast, the low cytotoxicity levels in all the test groups at these time points might be attributed to the early death of a great amount of SaOS-2 once in contact with CHX-based agents.

Caspase activation is considered a hallmark of programmed death, or apoptosis [38]. In the current study, caspase-3/7 substrates were utilized for the detection of the activity of these two effector caspases. In a previous study of this group [20], utilizing the same assessment method and cell line (i.e., SaOS-2) to test the in vitro properties of antimicrobial agents, a different control was adopted, i.e., pure water instead of NaCl. Regardless of the type of control, in both studies, higher apoptosis values were detected in the presence of the control compared to CHX-based ones. The low apoptotic levels registered among the test groups might be attributed to the dominant cytotoxic effect of the mouthwashes, while the results detected in the control group might be due to common environmental stress, especially after longer culture time, related to cell confluence, increased amount of waste products and reduced nutrition medium [39].

A fundamental condition for the successful treatment of peri-implantitis is the re-osseointegration of the implants. One of the determinant factors influencing this process, i.e., the re-osseointegration, consists in the effective decontamination of dental implants. To this aim, several approaches have been proposed, with no proven long-term clinical advantage of one method over the others [5,40]. Chemical agents can be used intraoperatively, alone or in combination with other methods, to eliminate bacterial biofilm adhering to the exposed implant surfaces. Indeed, peri-procedural rinsing with CHX has also been recommended for implant surgery in order to reduce the bacterial load [41]. Furthermore, rinsing with CHX after periodontal and implant surgery has been correlated with a significant reduction in plaque and bleeding as compared to placebo [42]. Besides their antimicrobial properties, these products should not exert a detrimental effect on the surround-

ing tissues, and eventual residues should not compromise the cellular response to the decontaminated surfaces [43]. Re-osteointegration largely depends on the initial cell response at the cell-implant interface. The main cells responsible for new bone apposition are osteoblasts and their precursors; therefore, assessing the effect of different mouthwashes on these cells is particularly relevant for the proposed clinical application.

Prior in vitro research has demonstrated the cytotoxicity of CHX on both osteoblastic and osteoblastic-like (e.g., SaOS-2) cell lines. In a previous paper by our group using a similar study design [20], at day 0, CHX 0.2 exhibited the highest cytotoxicity on SaOS-2, especially after 120 s of exposure, with significantly higher values compared to the taurolidine 2% and the pure water group. In Giannelli et al. [26], exposure to CHX induced a decrease in SaOS-2 cell viability in a dose- and time-dependent manner, while in the present study, the differences between the groups (different CHX concentration/application time) were not pronounced. Similar to our investigation, CHX-based mouthwashes were able to induce both apoptosis and necrosis. The treatment with 0.2% CHX also induced a drastic reduction of viability of both SaOS-2 and bone marrow mesenchymal stromal cells seeded onto titanium disks as compared to untreated cells [44]. Interestingly, CHX-induced cell damage resulted in being attenuated by rinsing with PBS, and even more if followed by air drying. In Vörös et al., 0.1% CHX was found to cause cell damage on human osteoblasts already after an incubation time of one minute [25]. The viability of murine osteoblast precursor cells significantly decreased when exposed to 0.12% CHX as compared to the control, irrespectively of the application time ranging from 30 s to 4.5 min [45].

The cytotoxic profile of CHX was also corroborated at lower concentrations. Osteoblast survival rate 48 h after an exposure to CHX (0.002%) was significantly reduced as compared to the control for all the exposure times (i.e., 1 m, 2 m and 3 m) (Liu et al., 2018). The low viability levels registered in this last work could have been ascribed to the relatively short culture time, masking the regenerative capacity of the cells over time. Therefore, in a recent study investigating the effect of different antiseptic solutions, a longer observation time was selected as in our paper [46]. The cytotoxic effect of CHX was confirmed also at a low concentration (0.05%), with human osteoblast cells failing to recover over the course of 5 days.

To the best of our knowledge, no other paper has previously investigated the effect of a 0.05% CHX + 0.05% CPC solution on osteoblast-like cells in vitro. However, the current study presents some limitations. Firstly, it was confined to a laboratory setting and the obtained results may not correspond to the oral environment, as a monolayer cell culture model cannot fully represent the bone tissue exposure to the antiseptic agents. Osteoblast-like cells were here directly exposed to the mouthwashes, while in vivo they reside within the mineralized bone tissue, which may reduce the permeability and the adsorption of the chemicals. Many aspects cannot be investigated in vitro, including the dilution of the mouthwashes in the fluids present in the oral cavity, the immunological response of the organism, as well as the tissue alterations resulting from the pathology itself [25]. In the present work, a two-dimensional (2D) system was chosen due to the high reproducibility of the experimental results and the ease of culture maintenance. Nevertheless, the morphology as well as the functions of cells grown as a monolayer attached to a glass or plastic surface resulted in being altered compared to those in the natural environment [47,48]. Despite the higher costs and technical difficulties, three-dimensional (3D) cell culture models have gained increasing interest owing to their closer resemblance to the in vivo microenvironment [47,49]. Furthermore, bone repair is a complex process which involves the well-orchestrated interactions between different cells and signals [50]. Microvascular circulation is considered a key component during tissue repair, and the lack of angiogenesis or its inhibition has been reported to hamper bone healing [51]. Newly formed vessels not only supply nutrients and oxygen to meet the local metabolic demands, but also produce inflammatory and injury-induced angiocrine signals, which con-

tribute to guiding bone regeneration [52]. Therefore, 3D co-cultures of osteoblasts and endothelial cells or concurrent multi-lineage differentiation of stem cells might be considered for future studies, prior to in vivo preclinical investigations or human clinical trials.

It is worth mentioning that human tissues usually present a higher tolerance for antiseptic agents compared to monolayer tissue cultures [24]. Indeed, higher regenerative potential is observed in vivo, where the recruitment of osteoprogenitors, hematopoietic stem cells and immune cells plays a fundamental role in tissue regeneration and remodeling [50]. Moreover, the fast growing of cells on a plastic support may further contribute to cell damage, as testified by the high cytotoxicity and apoptotic values reported in the control group at day 3 and 6. When resective surgical treatment of peri-implantitis was combined with surface decontamination with a 0.12% CHX + 0.05% CPC solution, a reduction of the anaerobic bacterial load was observed as compared to a placebo solution [33]. The significant reduction in bacterial load did not translate into an overall clinical or radiographical benefit. However, no detrimental effect was associated with the antiseptic agent. As a consequence, a CHX + CPC solution containing an even lower concentration of CHX could represent a safe antiseptic for this specific application.

Finally, implant surface characteristics have been demonstrated to affect cell response. Therefore, it would be interesting to investigate the effect of the mouthwashes on cells seeded onto different implant surfaces. Pre-treatment of the implant surfaces, simulating commonly applied clinical procedures such as implantoplasty, and different rinsing times with PBS or water after mouthwash application might also be determinant.

All of the mouthwashes tested here caused irreversible SaOS-2 cell damage, as confirmed by the low viability values and the respective low cytotoxicity and apoptotic levels registered at day 3 and 6. The main differences among the tested treatment procedures were observed at day 0, when overall the CHX 0.2 solution was found to exert a higher cytotoxic effect as comparted to the other mouthwashes. While a time-related effect on cell recovery and death was not noticed in the majority of the cases in all the experiments, at day 0 shorter application times were associated to lower cell cytotoxicity in both the CHX 0.1 and CHX + CPC group. It can be deduced that both these products could be considered for intraoperative usage, especially for a short rinsing time, while long application time and the exposure to CHX at the standard concentration of 0.2% should be avoided. How the present findings could be translated into a clinical situation remains to be clarified.

4. Materials and Methods

4.1. Cell Culture

Osteoblast-like cells (SaOS-2 cells) were seeded on sterile 96-well binding cell-culture plates (Costar 9102, Kennebunk, USA). Following the protocol described in John et al. (John et al., 2014), 10,000 SaOS-2 cells (Acc 243, fourth passage, German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) were cultured for 3 days in 200 µL of high-glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Merck Group, St. Louis, MO, US) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco Invitrogen, Darmstadt, Germany) at a temperature of 37 °C, 95% of humidity and 5% CO_2.

4.2. Treatment Procedure

After 3-day cell culture, a total of 288 wells were randomly assigned to the following treatment groups: 0.05% CPC + 0.05% CHX (PERIO-AID® Active Control, Dentaid® GmbH, Barcelona, Spain) (CPC + CHX), 0.1% CHX (Chlorhexamed® Fluid 0.1%, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG, Bühl, Germany), 0.2% CHX (Chlorhexamed® Forte 0.2%, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG), and sterile saline (NaCl) as control. In the attempt to replicate in vitro the situation of a mouthwash, nutrition medium was removed before the treatment and cells were gently rinsed

with phosphate buffered saline (PBS, Sigma-Aldrich). Three treatment times (i.e., 30, 60 and 120 s) were tested in each of the four groups.

Test and control mouthwashes were removed, the wells were gently rinsed with PBS and 200 μL of high-glucose DMEM was applied per well. Two hours (day 0), 3 days and 6 days after the treatment procedure with the mouthwashes, cell viability, cytotoxicity and apoptosis were assessed. In the 6-day groups, the nutrition medium was changed at day 3. Before performing the tests, the nutrition medium was removed and the wells were gently rinsed with PBS.

For each application time and assessment time point, 8 wells per product were examined.

4.3. Cell Viability, Cytotoxicity and Apoptosis

The effect of different treatment procedures on cell viability, cytotoxicity and apoptosis was determined by means of a triplex assay (ApoTox-Glo™ Triplex Assay, Promega, Mannhein, Germany) following manufacturer's instructions.

Firstly, cell viability and cytotoxicity were assessed simultaneously by fluorometry, measuring two protease activities. A viability/cytotoxicity reagent, containing both glycyphenylalanyl-aminofluorocoumarin (GF-AFC) and bis-alanylalanyl-phenylalnyl-rhodamine 100 (AAF-R110), was utilized. GF-AFC is a cell-permeant peptide which enters intact living cells where it is converted into amino fluorocoumarin (AFC), generating a fluorescent signal proportional to the amount of living cells. AAF-R110 is a cell-impermeant peptide, which is converted by dead-cell protease in rhodamine 110 (R100), when the protease is released in the culture medium due to the loss of cell membrane integrity. The metabolic products can be detected simultaneously, owing to the different mission spectra (AFC in green and R110 in red). Thereafter, for apoptosis, caspase-3/7 activity was measured by adding a luminogenic caspase-3/7 substrate, which can be evaluated via the production of a luminescent signal proportional to the amount of caspase activity present.

All signal measurements were performed using a luminometer/fluorometer (Victor 2030, PerkinElmer, Rodgau, Germany). Results were expressed in counts per second (CPS).

4.4. Statistical Analysis

Statistical evaluation was performed using the software R [53]. For each time point, application time and mouthwash, boxplots were created for descriptive purposes. The Kruskal–Wallis test, post hoc multiple comparison test and Bonferroni method for p-value adjustment were used to assess statistical differences in cell viability, cytotoxicity and apoptosis among the three treatment groups per time point, and adjusted p-values were reported. The results were considered significant at $p < 0.05$.

5. Conclusions

Further studies are needed to determine the impact of the different products and rinsing times on wound healing when they are used intraoperatively, in direct contact with the bone. Besides the safety of the rinsing procedure, their efficacy in terms of bacterial load reduction, improved bone healing and decreased peri-implantitis recurrences should also be investigated. It would also be important to evaluate the clinical effects of peri-incisional rinsing and postoperative dressings containing CHX-based solutions.

Future research could also be tailored to the investigation of different rinsing protocols in similar contexts, such as extraction socket rinsing or other surgical procedures in which a full thickness mucoperiosteal flap is raised, exposing the bone to the oral cavity.

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4. Original work

applied sciences

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Article

Effect of Three Chlorhexidine-Based Mouthwashes on Human Gingival Fibroblasts: An In Vitro Study

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Abstract: Mouthwashes containing chlorhexidine (CHX) are deemed to be associated with dose-dependent side effects, including burning sensation and taste alteration. To overcome these drawbacks, mouthwashes with CHX at lower concentrations with or without adjunctive agents are proposed. The aim of this in vitro study was to investigate the effects of three CHX-based mouthwashes on human gingival fibroblasts (HGFs). After 3 days of cell culture, groups were randomly treated for 30 s, 60 s or 120 s with (a) CHX 0.05% in combination with cetylpyridinium chloride (CPC) 0.05%; (b) CHX 0.1%; (c) CHX 0.2%; or (d) NaCl as control. Cell viability, cytotoxicity and apoptosis were evaluated at 2 h, 3 days and 6 days after the exposure to the different solutions. Similar cell viability values were found among the test groups at all time points. At day 0, higher cytotoxicity was measured in the group treated with CHX 0.2%, in particular after long application time (120 s), while no significant difference was found between CHX + CPC and the control group. All the investigated mouthwashes were well tolerated by HGF cells for the tested application times. The highest cytotoxic effect was observed for CHX 0.2%; therefore, clinicians should consider limiting its usage to carefully selected clinical situations.

Keywords: antiseptic; apoptosis; cetylpyridinium chloride; mouthrinse

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1. Introduction

Oral biofilm is considered the principal etiologic factor responsible for the onset, the development and the recurrence of periodontitis and peri-implantitis [1–6]. Furthermore, tissue healing can be impaired by the presence and accumulation of oral biofilm after the surgical treatment of periodontal and peri-implant diseases, when effective mechanical self-care cannot be adequately performed [7]. Thus, plaque control is deemed to be essential for both the recovery and the maintenance of healthy tissue conditions [7,8].

In adjunction to professional mechanical debridement, adequate self-administered daily home care is fundamental for the long-term success of the treatments [8–10]. At-home measures frequently include the use of antiseptic mouthwashes. In addition to adequate antibacterial activity, these products should not trigger any allergic reactions or provoke tissue damage [11,12].

Concerns may arise in cases of prolonged usage or when the antimicrobial agent comes in direct contact with the connective tissues, for instance during postoperative wound healing [11,13]. In vitro assays are frequently utilized for analyzing the cytotoxicity of antiseptic agents as well as of some filling resin frequently used dental materials, owing

to their reduced costs and their high repeatability and reproducibility [14]. In particular, human gingival fibroblasts are commonly used to mimic connective tissue exposure to mouthwashes and to investigate cell-induced stress [15–19].

Chlorhexidine (CHX), a bisbiguanide broad-spectrum antiseptic, has been widely used for chemical plaque control [20]. However, it is well documented that the prolonged use of mouthwashes with CHX at high concentration can lead to several undesired side effects, including tooth staining, taste alteration and burning sensation [20–23]. CHX-based mouthwashes on the market are generally at a concentration of 0.1%, 0.12% or 0.2% CHX digluconate, or they present a low concentration equal to or below 0.06% [20]. Research has tended towards the formulation of mouthwashes presenting lower cytotoxicity, while maintaining high antibacterial properties. To overcome the aforementioned drawbacks, mouthwashes containing low concentrations of CHX, alone or in combination with additional compounds, have been proposed [20]. Among them, cetylpyridnium chloride (CPC) seems to be particularly promising [24–28]. CPC is an amphiphilic cationic quaternary ammonium compound, whose antimicrobial activity is mainly related to its capability to bind to and destroy the bacterial cell membrane. Whereas, at low concentrations, it indirectly promotes cell autolysis through the activation of intracellular latent ribonucleases [29]. Several mouthwash formulations containing both CHX and CPC have been investigated, including solutions with CHX at low concentration, such as CHX 0.05 % + CPC 0.05 % [26,30,31] or CHX 0.03 % + CPC 0.05 % [24,25], but also at higher concentration (e.g., CHX 0.12 % + CPC 0.05 %) [32].

In a recent study by our group, a CHX 0.05 % + CPC 0.05 % was found to be effective against oral bacteria in vitro; however, limited data on its cytotoxicity are available [30]. Therefore, the aim of the present in vitro study was to investigate the effects of three mouthwashes containing CHX at different dilutions, alone or combined with CPC, on human gingival fibroblasts (HGFs) by examining cell viability, cytotoxicity, and apoptosis after 0, 3 and 6 days from the exposure. The null hypotheses were that, at the three time points, there would be no significant difference among groups in cell viability, cytotoxicity and apoptosis.

2. Materials and Methods

The current in vitro study was reported in accordance with the modified Consolidated Standards of Reporting Trials (CONSORT) guidelines [33].

2.1. Cell Culture

Two hundred eighty-eight wells were seeded with human gingival fibroblasts (HGFs), using 96-well binding plates (Costar® 9102, Corning, New York, US). As previously described [16], 5000 HGFs (HGFIB, passage 5, Provitro AG) per well were cultured for 3 days in 200 µL of high-glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, Missouri, US), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco, Invitrogen, Waltham, MA, USA) at 37 °C, 5% CO_2, and 95% humidity.

To simulate oral rinse, the culture medium was carefully aspirated, and cells were gently rinsed with phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) before treatment.

2.2. Treatment Procedure

Following cell culture, wells were randomly assigned to four different groups: (a) CPC 0.05% + CHX 0.05% (PERIO-AID® Active Control, Dentaid® GmbH) (regarded as CPC + CHX); (b) CHX 0.1% (Chlorhexamed® Fluid 0.1%, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG) (regarded as CHX 0.1); (c) CHX 0.2% (Chlorhexamed® Forte 0.2%, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG) (regarded as CHX 0.2); (d) control, i.e., sterile saline (regarded as NaCl). Three treatment times (i.e., 30 s, 60 s and 120 s) were tested in each group. The mouthwashes were removed, the wells were gently rinsed with PBS

and 200 µL nutrition medium was added (high-glucose DMEM). The measurements were conducted after 2 h (day 0), 3 days and 6 days following the treatment with mouthwashes. The culture medium was refreshed at day 3 in the 6-day groups. Before carrying out the experiments, the culture medium was removed, and the wells were gently washed with PBS. Figure 1 shows the flowchart of the study.

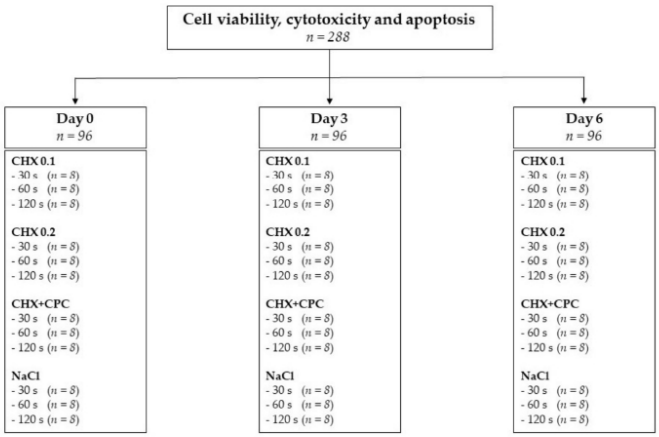

Figure 1. Flowchart of the study. The number of wells utilized for each time point and mouthwash is indicated in brackets (n =).

2.3. In Vitro Tests

Cell viability, cytotoxicity and apoptosis were measured using a single luminescence assay (ApoTox-Glo™ Triplex Assay, Promega, Fitchburg, Wisconsin, US) in a luminometer/fluorometer (Victor 2030, PerkinElmer, Waltham, Massachusetts, US). The measurements are reported in counts per second (CPS). This experiment is characterized by two consecutive phases. First, cell viability and cytotoxicity were simultaneously assessed by fluorometry, measuring two protease activities. The live-cell protease activity was measured using glycyl-phenylalanyl-amino fluorocoumarin. This is a cell-permeant peptide, which enters intact living cells, where it is converted into amino fluorocoumarin (AFC). The resulting fluorescent signal is proportional to the amount of living cells. Cytotoxicity was determined using a fluorogenic cell-impermeant peptide (i.e., bis-alanyl-alanyl-phenylalanyl- rhodamine 110). It is converted by dead-cell protease in rhodamine 110 (R100), which is released only by cells that have lost their membrane integrity. AFC and R110 were detected simultaneously, due to the different mission spectra (green and red, respectively). In the second part of the assay, analysis was performed to determine whether the investigated solutions could cause apoptosis. This was measured using a luminogenic caspase-3/7 substrate. Luminescence was proportional to the degree of caspase activity present.

2.4. Statistical Analysis

Data analysis was performed utilizing the free software R [34]. A sample of convenience was used. Boxplots were created for descriptive purposes for each selected variable. To determine the presence of any significant difference in cell viability, cytotoxicity and apoptosis among the three treatment groups per time point, a Kruskal–Wallis test with post hoc multiple comparison test with the Bonferroni method for p-value adjustment was used, and adjusted p-values were reported. A p-value < 0.05 was considered statistically significant.

3. Results

The results of cell viability, cytotoxicity and apoptosis of HGFs are presented in Figures 2–4. No sign of bacterial or fungal contamination was observed along the entire experimental period.

3.1. Cell Viability

An overview of cell viability results is presented in Figure 2. The highest values were predictably found in the NaCl group for all time points and exposure times to the mouthwashes. Against our expectations and not in line with the graph (Figure 2), no significant difference was shown between NaCl and CHX + CPC groups at day 0 (120 s), at day 3 (30 s, 60 s, and 120 s), and at day 6 (60 s and 120 s). Moreover, no significant difference was identified between NaCl and CHX 0.2 at day 6 (30 s) (Table 1). The significance level reported in Table 1 is after Bonferroni correction. The uncorrected *p*-values were <0.05 in all these cases but one (CPC + CHX vs. NaCl 60 s at day 6: $p = 0.07$).

After 3 days of culture, the CHX + CPC group exhibited significantly higher cell viability compared to the CHX 0.1 group for both 60 s and 120 s application times; similarly, on day 6 this was observed after a treatment time of 60 s (Table 1).

Table 1. Cell viability. A multiple Kruskal–Wallis test was performed to compare the groups at 0, 3 and 6 days. In case of significance, a post hoc test was performed, and the adjusted *p*-values from the post hoc test are reported here.

Grouping Variable	Comparator 1	Comparator 2	*p*-Value (day 0)	*p*-Value (day 3)	*p*-Value (day 6)
CHX 0.1	30 s	60 s	-	-	0.442
	30 s	120 s	-	-	0.143
	60 s	120 s	-	-	0.002 **
CHX 0.2	30 s	60 s	-	-	0.002 **
	30 s	120 s	-	-	0.143
	60 s	120 s	-	-	0.442
CHX + CPC	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
NaCl	30 s	60 s	1.000	-	1.000
	30 s	120 s	0.049 *	-	0.009 **
	60 s	120 s	0.022 *	-	0.033 *
30 s	CHX 0.1	CHX 0.2	1.000	1.000	0.420
	CHX 0.1	CHX + CPC	1.000	0.151	1.000
	CHX 0.1	NaCl	0.016 *	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.000 ***	0.001 **	0.106
	CHX + CPC	NaCl	0.007 **	0.226	0.001 **
60 s	CHX 0.1	CHX 0.2	1.000	0.373	1.000
	CHX 0.1	CHX + CPC	1.000	0.020 *	0.046 *
	CHX 0.1	NaCl	0.022 *	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	1.000	1.000	0.198
	CHX 0.2	NaCl	0.000 ***	0.010*	0.000 ***
	CHX + CPC	NaCl	0.005 **	0.226	0.420
120 s	CHX 0.1	CHX 0.2	1.000	0.092	1.000
	CHX 0.1	CHX + CPC	1.000	0.043 *	0.445
	CHX 0.1	NaCl	0.008 **	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	0.420	1.000	1.000
	CHX 0.2	NaCl	0.000 ***	0.043 *	0.003 **
	CHX + CPC	NaCl	0.054	0.092	0.079

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

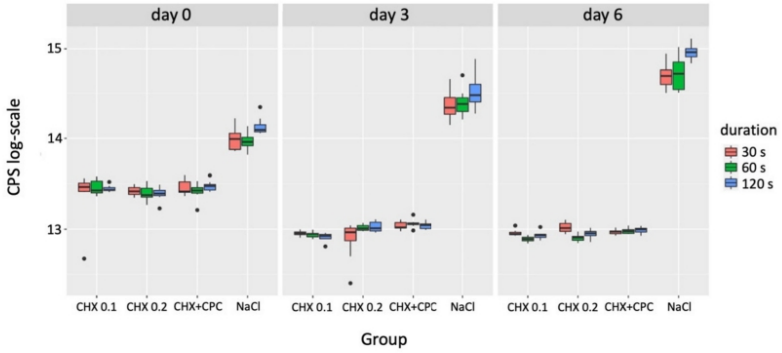

Figure 2. Overview of cell viability of HGFs after exposure to test and control mouthwashes (for 30 s, 60 s and 120 s) measured in CPS at day 0, 3 and 6.

3.2. Cytotoxicity

On day 0, the CHX 0.2 groups exhibited the highest cytotoxicity values, especially with the longest application time. Whereas, after both 3 and 6 days of culture, the highest values were registered in the NaCl group, as it clearly emerges from the boxplot (Figure 3). However, as for the cell viability assay, no significant difference was identified between NaCl and CHX + CPC groups at day 3 (30 s, 60 s and 120 s) and day 6 (60 s and 120 s). The significance level reported in Table 2 is after Bonferroni correction. The uncorrected *p*-values were <0.05 in all these cases but one (CPC + CHX vs NaCl 30 s at day 3: $p = 0.058$).

Interestingly, at day 0, after 120 s treatment time, CHX 0.2 showed significantly higher cytotoxicity not only compared to the control (NaCl) but also to the CHX + CPC group. Regarding the application time, CHX 0.2 was significantly more cytotoxic once applied for 120 s than for the short treatment time (30 s) at both day 0 and 3.

Interestingly, no significant differences could be identified on day 0 between the CHX + CPC and NaCl group for all the application times (30 s, 60 s and 120 s) (Table 2). Moreover, at day 3, significant higher values were observed in CHX + CPC than in the CHX 0.1 group after both 60 s and 120 s of treatment.

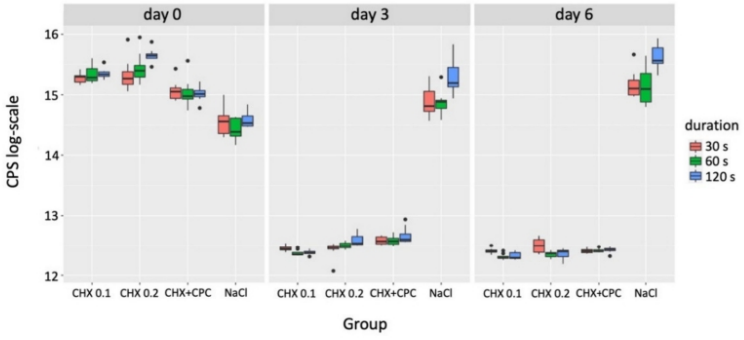

Figure 3. Overview of cytotoxicity of HGFs after exposure to test and control mouthwashes (for 30 s, 60 s and 120 s) measured in CPS at day 0, 3 and 6.

Table 2. Cytotoxicity. A multiple Kruskal–Wallis test was performed to compare the groups at 0, 3 and 6 days. In case of significance, a post hoc test was performed, and the adjusted *p*-values from the post hoc test are reported here.

Grouping Variable	Comparator 1	Comparator 2	*p*-Value (day 0)	*p*-Value (day 3)	*p*-Value (day 6)
CHX 0.1	30 s	60 s	-	0.014 *	0.030 *
	30 s	120 s	-	0.121	0.049 *
	60 s	120 s	-	1.000	1.000
CHX 0.2	30 s	60 s	1.000	0.609	0.040 *
	30 s	120 s	0.024 *	0.006 **	0.231
	60 s	120 s	0.269	0.214	1.000
CHX + CPC	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
NaCl	30 s	60 s	-	1.000	1.000
	30 s	120 s	-	0.024 *	0.027 *
	60 s	120 s	-	0.024 *	0.016 *
30 s	CHX 0.1	CHX 0.2	1.000	1.000	1.000
	CHX 0.1	CHX + CPC	0.292	0.099	1.000
	CHX 0.1	NaCl	0.000 ***	0.000 ***	0.001 **
	CHX 0.2	CHX + CPC	0.373	0.185	1.000
	CHX 0.2	NaCl	0.001 **	0.000 ***	0.033 *
	CHX + CPC	NaCl	0.257	0.351	0.002 **
60 s	CHX 0.1	CHX 0.2	1.000	0.420	1.000
	CHX 0.1	CHX + CPC	0.420	0.017 *	0.073
	CHX 0.1	NaCl	0.001 **	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	0.173	1.000	0.814
	CHX 0.2	NaCl	0.000 ***	0.008 **	0.002 **
	CHX + CPC	NaCl	0.226	0.257	0.226
120 s	CHX 0.1	CHX 0.2	0.591	0.185	1.000
	CHX 0.1	CHX + CPC	0.472	0.019 *	0.226
	CHX 0.1	NaCl	0.004 **	0.000 ***	0.000 ***
	CHX 0.2	CHX + CPC	0.004 **	1.000	1.000
	CHX 0.2	NaCl	0.000 ***	0.019 *	0.002 **
	CHX + CPC	NaCl	0.591	0.185	0.141

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. Apoptosis

Overall, the highest values were registered in the control group (NaCl) at all time points (Figure 4). In the control group, significant differences were detected between 120 s and the other application times in all cases but one (60 s vs. 120 s, day 3). Whereas, within each test group, the exposure time to the mouthwashes had no significant effect on HGF apoptosis at all time points (Table 3).

As above, contrary to our expectations and not in line with the graphical illustration (boxplot, Figure 4), no significant difference was detected between NaCl and CHX 0.1 groups at day 6 (30 s and 60 s), as well as between the NaCl and CHX 0.2 groups at day 0 (120 s). The significance level reported in Table 3 is after Bonferroni correction. The uncorrected *p*-values in all these cases were <0.05.

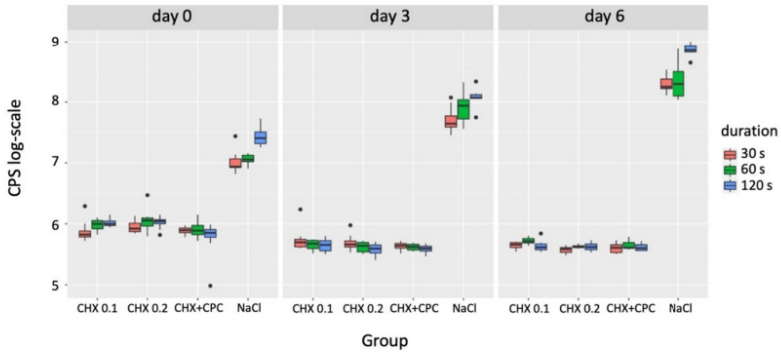

Figure 4. Overview of apoptosis of HGFs after exposure to test and control mouthwashes (for 30 s, 60 s and 120 s) measured in CPS at day 0, 3 and 6.

Table 3. Apoptosis. A multiple Kruskal–Wallis test was performed to compare the groups at 0, 3 and 6 days. In case of significance, a post hoc test was performed, and the adjusted *p*-values from the post hoc test are reported here.

Grouping Variable	Comparator 1	Comparator 2	*p*-Value (day 0)	*p*-Value (day 3)	*p*-Value (day 6)
CHX 0.1	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
CHX 0.2	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
CHX + CPC	30 s	60 s	-	-	-
	30 s	120 s	-	-	-
	60 s	120 s	-	-	-
NaCl	30 s	60 s	1.000	0.648	1.000
	30 s	120 s	0.002 **	0.006 **	0.004 **
	60 s	120 s	0.011 *	0.183	0.009 **
30 s	CHX 0.1	CHX 0.2	0.813	1.000	0.623
	CHX 0.1	CHX + CPC	1.000	1.000	1.000
	CHX 0.1	NaCl	0.000 ***	0.016 *	0.065
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.048 *	0.004 **	0.000 ***
	CHX + CPC	NaCl	0.004 **	0.001 **	0.003 **
60 s	CHX 0.1	CHX 0.2	1.000	1.000	0.291
	CHX 0.1	CHX + CPC	1.000	1.000	0.444
	CHX 0.1	NaCl	0.007 **	0.023 *	0.185
	CHX 0.2	CHX + CPC	1.000	1.000	1.000
	CHX 0.2	NaCl	0.028 *	0.002 **	0.000 ***
	CHX + CPC	NaCl	0.000 ***	0.001 **	0.000 ***
120 s	CHX 0.1	CHX 0.2	1.000	1.000	1.000
	CHX 0.1	CHX + CPC	0.248	1.000	1.000
	CHX 0.1	NaCl	0.032 *	0.024 *	0.004 **
	CHX 0.2	CHX + CPC	0.167	1.000	1.000
	CHX 0.2	NaCl	0.052	0.001 **	0.005 **
	CHX + CPC	NaCl	0.000 ***	0.002 **	0.003 **

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

The purpose of the present in vitro study was to assess the possible effects of three commercially available CHX-based mouthwashes on human gingival fibroblasts (HGFs). The null hypotheses were that there would be no significant difference among the groups in cell viability, cytotoxicity and apoptosis. They were partially rejected, and the corresponding alternatives were accepted.

Cell viability, as expected, was generally higher in the saline control group (NaCl) as compared to the use of mouthwashes, with similar values among the latter at all time points. Furthermore, cell viability was found not to be influenced by mouthwash application time in the large majority of cases.

Insights into the cellular death mechanisms provoked by the different treatment procedures were also uncovered. Despite the modifications of the plasma membrane in the final stages of apoptotic cell death, the rupture and the integrity of the cell membrane are generally considered as main features of necrosis and apoptosis, respectively [35,36].

Cytotoxicity was analyzed by measuring a proteolytic biomarker dependent on cell membrane disruption. In agreement with cell viability findings, at day 0, all the mouthwashes presented higher values as compared to the control. Interestingly, at this time point, CHX 0.2 exhibited significantly higher cytotoxicity compared to the CHX + CPC group after the long application time (120 s) and was significantly more cytotoxic once applied for 120 s than for the short treatment time (30 s). Moreover, at day 0, no statistically significant differences could be observed between CHX + CPC and the control group for all the application times. By contrast, at day 3, NaCl showed the highest cytotoxicity; significant differences were also observed between CHX + CPC and CHX 0.1 groups after both 60 s and 120 s of treatment. It can be speculated that CHX-based mouthwashes induced HGF death immediately after exposure, when the phenomenon could clearly be observed. Since most of the cells were likely to be dead in the early phases after the contact with the mouthwashes, in particular at higher CHX percentages, their cytotoxicity values drastically decreased already at day 3. Whereas, in the NaCl group, a balance between living and dead cells was maintained up to day 6 of culture.

Caspases are deemed to be responsible for the proteolytic cleavages leading to cell disassembly, which is typical of apoptosis [37]. Therefore, a luminescent assay measuring the activity of two effector caspases, which are expressed and activated in apoptotic cells, was here utilized. In accordance with a previous investigation of our team [16], the control group exhibited significantly higher values of apoptosis as compared to the test groups. The predominant cytotoxic action exerted by the mouthwashes could be an explanation for the low apoptosis values. Although optimal culture conditions were provided through constant cell coverage by culture medium, in the NaCl group, apoptosis values tended to increase over time; this might be ascribed to environmental stress, which can result from changes in cell density, nutrient depletion, or waste product accumulation [38].

For all the selected parameters, changes were mainly observed between day 0 and day 3, while the values remained almost unmodified from day 3 to day 6. As the majority of the events took place in the early phases after mouthwash application, it would be interesting to map and quantify these dynamic processes in real time and over time by means of live cell imaging [39,40].

Mouthwashes are widely used concomitant with periodontal and peri-implantitis treatments [8,41,42]. As these pathological conditions are associated with bacterial biofilm formation, antimicrobial properties are of major importance for supragingival plaque control [11,43]. Owing to its well-documented antibacterial activity, CHX is frequently employed to reduce oral bacterial load [20]. However, mouthwashes containing a high percentage of CHX have been associated with cytotoxic effects in vitro [12,16,44,45]. Therefore, usage of lower concentrations of CHX in combination with CPC have been proposed [24–28]. In a recent in vitro study by our group, a CHX 0.05% + CPC 0.05% mouthwash was revealed to be effective against oral living bacteria after in situ plaque accumulation, showing similar properties as comparted to CHX 0.1% solution [30]. Furthermore, utilizing the same study

design of the present work, the authors found the highest cytotoxicity on osteoblast-like cells at day 0 in the CHX 0.2% group, which also presented significantly higher values compared to CHX 0.05% + CPC 0.05% for all the application times [31]. Due to the limited data available on the cytotoxicity on fibroblasts of the former, the current work was conceived as a complementary study in support of our recent investigations [30,31]. In addition to the NaCl and CHX 0.1 groups, it was decided to add the CHX 0.2 group, as it still represents a commonly used solution, in particular after periodontal and peri-implantitis surgeries. In such cases, the protective epithelial barriers would no longer inhibit the direct contact between the connective tissue and the mouthwash; therefore, the cytotoxicity of the antiseptic agent should be carefully considered [11,13].

A limitation of this study is that it was based on monolayer cultures. Two-dimensional (2D) cell cultures were chosen due to the relatively easy environmental control and cell observation, which allow for the minimizing of measuring errors. Flat cultures are, indeed, considered particularly suitable for preliminary toxicity tests, but three-dimensional (3D) human oral mucosal models might be considered for further studies, due to their closer resemblance to the complex in vivo tissue microenvironment [46–49]. As the oral mucosa is characterized by multiple layers, in 3D models the direct contact of the fibroblasts with the mouthwashes can be avoided, better mimicking the tissue permeability of the agents through the epithelial outer layer [47]. This would be particularly relevant for translating the data to the chronic usage of mouthwashes, which might affect oral mucosa health in the long term [50].

Our results mark out a starting point for future clinical investigations, aiming at understanding not only the impact of different mouthwashes, but also the influence of different rinsing regimens on oral mucosa health and periodontal and peri-implant disease control.

In summary, the results obtained in the present study showed that the three tested CHX-based mouthwashes had similar effects on in vitro HGF viability, thus rejecting the null hypotheses that assumed no significant differences per time point and variable. At day 0, the CHX + CPC group presented milder cytotoxic effects as compared to the CHX 0.2 group after an application time of 120 s. In addition, at this time point, no significant differences could be identified between the CHX + CPC and the NaCl control group for all the application times, thus confirming the relatively moderate cytotoxicity of the CHX 0.05% + CPC 0.05% mouthwash.

Although extrapolating in vitro data to predict side effects in patients remains difficult, rinsing regimens should be carefully considered by the clinicians balancing the risks of cytotoxicity and the required antimicrobial effect in specific clinical circumstances.

High concentration of CHX might have detrimental effects on oral mucosa, not only in the case of prolonged usage, but also when applied directly in contact with connective tissues during wound healing.

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ORIGINAL ARTICLE

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Two-piece zirconia implants in the posterior mandible and maxilla: A cohort study with a follow-up period of 9 years

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Abstract

Objectives: Long-term follow-up observations of zirconia implants are rare. This study aimed at evaluating the clinical performance of two-piece zirconia implants in the posterior jaws over 9 years.

Materials and Methods: Sixty partially edentulous patients were treated with two-piece zirconia implants. In eight no primary stability could be achieved. Fifty-two patients received the final restoration (i.e., cemented fibreglass abutments and all-ceramic crowns). After 2 years, 2 implants failed and 4 dropouts were recorded. The remaining 46 patients with one target implant each were recalled at 9 years. Besides implant survival, clinical parameters at the implant level (plaque index–PI, bleeding on probing–BOP, probing depth–PD, mucosal recession–MR) were recorded and compared with previously collected data. Mechanical and technical complications were assessed.

Results: Thirty patients responded. The mean observation period was of 111.1 ± 2.2 months. One implant was lost. Data recorded from the remaining 29 implants were analysed. PI values increased overtime. Mean BOP and PD remained unchanged during follow-up. No additional cases of peri-implantitis were recorded over the 10 diagnosed during the first 2 years of follow-up. No significant changes in mean MR values were detected over time, with 65% of the all included implants exhibiting no recession at 9 years and all the others, but one, a maximum MR of 1 mm. Three technical and 6 mechanical complications occurred in 7 patients between 2- and 9-years (6.9% and 20.7%, respectively, at patient level).

Conclusion: Within the limitations of the present study, a high survival rate was registered. Albeit frequent mechanical and technical complications, two-piece zirconia implants could represent a valid solution for the replacement of single teeth in the posterior jaws.

KEYWORDS
clinical study, implant survival, zirconia implants

Frank Schwarz and Jürgen Becker contributed equally to this work.

1 | INTRODUCTION

Zirconia dental implants are regarded as a valid alternative to the commonly used titanium implants, owing to their high biocompatibility, favourable soft-tissue response, as well as tooth-like colour (Roehling et al., 2018). The spread of ceramic implants is projected to increase in the next decade (Kohal & Dennison, 2020; Sanz et al., 2019). This tendency can be ascribed to the current high levels of aesthetic expectation, as well as to the growing demands for metal-free solutions, at least among the European population (Cionca et al., 2017; Sanz et al., 2019).

Advancements in dental implant manufacturing have paved the way for the consolidation of high-strength ceramic materials in implant dentistry (Roehling et al., 2018). The first generation of ceramic implants was made of alumina (Al_2O_3). However, they are no longer available on the market due to their poor mechanical properties leading to a high rate of fracture at the implant neck (Cionca et al., 2017; Depprich et al., 2014). Since the beginning of the 90s, zirconia (ZrO_2) has been establishing itself as the material of choice for ceramic implants.

Zirconia is of particular interest for its excellent optical properties when used for transmucosal components (Bressan et al., 2011; Kniha et al., 2019; Kohal & Dennison, 2020). Aesthetic problems can be associated with the greyish shimmering of the titanium, which is not always masked by the surrounding soft tissues, especially in presence of a thin biotype (Jung et al., 2007; van Brakel et al., 2011). The transmucosal components play also a crucial role in the prevention of implant failure, as plaque accumulation and a weak mucosal seal around the implants may likely contribute to the onset of peri-implant diseases (Schwarz et al., 2018). Beside the noticeably enhanced appearance of the peri-implant tissues, zirconia surfaces have been demonstrated to be advantageous in terms of resistance to bacterial adhesion and colonization (Al-Radha et al., 2012; Rimondini et al., 2002; Scarano et al., 2004). Furthermore, it has been suggested that zirconia resulted in a stronger mucosal barrier at the soft-tissue implant interface (Kohal et al., 2004; Lee et al., 2019; Liñares et al., 2016; Welander et al., 2008).

Despite their favourable biological and aesthetic characteristics, the osseointegration of zirconia implants largely depends on the surface topography. Moderately rough surface-modified zirconia implants exhibited higher osteointegration properties than untreated ones, as well as similar or better outcomes compared to titanium implants (Depprich et al., 2008; Ding et al., 2020; Hafezeqoran & Koodaryan, 2017; Hempel et al., 2010; Kubasiewicz-Ross et al., 2018).

Among zirconia-based materials, yttria-stabilized tetragonal zirconia polycrystal (Y-TZP) has become quite popular for load-bearing applications, due to its ability to withstand occlusal loads (Roehling et al., 2018). It has to be noted that initial concerns regarding the fracture resistance of complex zirconia structures determined the development of implant systems characterized by a one-piece design. These implants are known to possess limited restorative flexibility and might be exposed to undesired immediate loading due to their conformation (Cionca et al., 2017; Payer et al., 2013; Pieralli et al., 2017). More recently two-piece zirconia implants were introduced in the commerce, thus overcoming the inherent limitations of one-piece implants. However, the late development of two-piece zirconia solutions reflects in the scarce information on their medium- and long-term clinical outcomes (Cionca et al., 2017; Pieralli et al., 2017; Roehling et al., 2018).

A previous prospective cohort study investigated the clinical performances of two-piece zirconia implants restored with cemented fibreglass abutments and all-ceramic single crowns in the posterior jaws (Becker et al., 2017). Despite 8 target implants out of 60 were lost due to the absence of primary stability and did not receive the final restoration, the short-term results on the remaining 52 were promising, with a cumulative survival rate of 95.8% (excluding early implant failures prior to loading), improved soft-tissue conditions and rare mechanical and technical complications over a period of 25 ± 5.8 months (Becker et al., 2017). The aim of the present study was to retrospectively evaluate the long-term clinical outcomes in the aforementioned patient cohort after a period of 9 years.

2 | MATERIALS AND METHODS

This study was designed as a single-centre cohort study. Patients received a detailed description of the procedure and gave their written informed consent to the treatment. The study was conducted in accordance with revised principles stated in the Helsinki Declaration and ethics approval for the follow-up assessments was obtained from the Ethics Committee of the Heinrich Heine University of Düsseldorf, Germany (Prot. Number 3712/2021). The study was reported in accordance to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for reporting observational studies (von Elm et al., 2014).

2.1 | Patient population and study design

The original population consisted of 60 partially edentulous patients in need for at least one single-tooth implant-supported fixed prosthesis in the premolar/molar regions of either the maxilla or the mandible. Details of the treatment protocol were reported previously (Becker et al., 2017). In brief, 60 patients received, between November 2011 and April 2012, two-piece, screw-type zirconia implants (Patent™, Zircon Medical, Altendorf, Switzerland—former ZV3, Zircon Vision GmbH, Wolfratshausen, Germany) with individualized heights of the transmucosal aspect (Figure 1). The implants had diameters of 4.5 and 5.0 mm and were used in three different lengths, that is 9, 11 or 13 mm. In case of multiple implant placements in the same patient, the most anterior site was considered as target as decided a priori in the original protocol. An insufficient primary implant stability could be achieved in eight cases (early implant failure prior to loading); therefore, only 52 patients out of 60 were restored with all-ceramic single crowns cemented on fibreglass

FIGURE 1 Schematic cross-section of the 2-piece zirconia implant, highlighting the three components, that is the ceramic implant, the cemented fibreglass abutment (light green) and the all-ceramic crown.

abutments using a conventional loading protocol. At 2-year follow-up, 2 target implants failed and 4 dropouts were recorded. The remaining 46 patients with one target implant each were recalled for the 9-year follow-up examination.

Inclusion criteria were as follows:

The Subjects were included in the study if they present all of the following conditions: (1) Successful implant placement in the initial study (Becker et al., 2017), (2) final restoration and (3) written informed consent.

The subjects were not included in the study if they present one of the following conditions: (1) occurrence of newly diagnosed diseases interfering with implant success, (2) history of a trauma to the implant site, (3) pregnant or lactating women, (4) participation in a clinical study interfering with the objective of this follow-up observation, (5) unregular maintenance care.

2.2 | Surgical procedure and prosthetic rehabilitation

All the surgeries were carried out under local anaesthesia by three experienced and previously calibrated oral surgeons. In brief, after the elevation of a mucoperiosteal flap, implant site preparation was performed under copious irrigation following the manufacturer's guidelines. Good primary stability, defined as absence of clinical implant mobility, had to be achieved and each customized implant had to be positioned as preoperatively planned, in a way so that the limit between the transmucosal and intrabony part of the implant coincided with the lingual bone crest. Implant diameter and length were selected based on the individual clinical and radiological situation. Simultaneous grafting of buccal dehiscence-type defects with deproteinized bovine bone mineral particles (Bio-Oss®, Geistlich Pharma AG, Wolhusen, Switzerland) and resorbable collagen membranes (Bio-Gide®, Geistlich Pharma AG) as well as transcrestal sinus lift were performed, if required. In cases of sinus lift using lateral window approach, implants were inserted after 4–6 months from grafting (Bio-Oss®, Bio-Gide®). One-stage implant placement was used in all cases with transmucosal healing and without any provisional restoration. Implant loading was accomplished after approximately 12 and 10weeks in the maxilla and in the mandible, respectively. Fibreglass abutments were cemented using a dual-cure resin cement and a self-adhesive primer (Panavia F2.0, Kuraray Europe GmbH, Hattersheim am Main, Germany). Then, conventional impressions using a monophase technique were taken with polyether material (Impregum, 3M Deutschland GmbH, Neuss, Germany) and monolithic all-ceramic single crowns (IPS e.max, Ivoclar Vivadent GmbH, Ellwangen, Germany) were fixed using the same cement.

2.3 | Supportive therapy

Individualized supportive care program included professional cleaning, local pocket irrigation using chlorhexidine and patients' motivation. Patients were recalled, depending on their individual needs, in the first two years from the therapy. Thereafter, the patients were under regular maintenance care either at the Department or at the referring dentist according to individual needs.

2.4 | Clinical examinations

At the baseline (i.e., crown delivery), and after 2 and 9 years, the following clinical parameters were recorded for each of the available target implants as described previously (Becker et al., 2017): (1) plaque index (PI), (2) bleeding on probing (BOP), (3) probing depth (PD) and (4) mucosal recession (MR) measured taking as fixed

reference point the crown margin, as each customized implant had been designed and manufactured in a way that the implant neck and subsequently the crown margin were located in an epimucosal position. At the 9-year follow-up, a dichotomous plaque index was used (O'Leary et al., 1972); therefore, the PI values (Löe, 1967) from previous examinations were modified accordingly, considering 0 as absence of plaque and values from 1 to 3 as presence of plaque. All measurements were performed at six aspects per implant: mesiobuccal (mb), midbuccal (b), distobuccal (db), mesiooral (mo), midoral (o) and distooral (do). All the measurements were performed by two investigators in the first two years, while two other investigators (N.R. and G.J.) collected the data at the 9-year follow-up. All examiners initially underwent a standard calibration procedure as required for clinical routine examinations in the authors' Department. This included double measurements of the assessed clinical parameters, which were commonly performed within a 5-minute interval in three patients and accepted when repeated measurements were similar at >95% level. Implant mobility (i.e., loss of osseointegration) was also recorded by manual palpation. According to the German Röntgenverordnung based on 97/43/EURATOM directive and the Strahlenschutzgesetz based on the 103/2013 Euratom directive, two-dimensional radiographs for the assessment of marginal bone level changes at 9 years were not routinely justified. This included suspected cases of peri-implant mucositis, as defined by Renvert et al. (2018), where the radiographic assessment would have not changed the therapeutic approach. Consequently, radiographs were taken if clinically justified (e.g., in presence of both BOP e PPD ≥ 6 mm or mechanical/technical complications).

2.5 | Survival and complications

Implant survival was considered as the presence of the implant in situ at the 9-year follow-up examination. Technical and mechanical complications occurred during the follow-up period were recorded. Technical complications comprised all the events affecting the cemented crown (according to the definition of Heitz-Mayfield et al., 2014) as well as the decementation of the fibreglass abutment. Mechanical complications were considered all the events affecting the integrity of the implant or of the abutment. Biological complications considered the presence of peri-implantitis at the target implant, as defined by Berglundh et al. (2018) (i.e., presence of bleeding and/or suppuration on gentle probing, probing depths of ≥6 mm and bone levels ≥3 mm apical of the most coronal portion of the intraosseous part of the implant) or of mucositis (Renvert et al., 2018).

2.6 | Statistical analysis

The statistical analysis was performed using R (R Core Team, 2021) and SPSS (IBM Corp., Armonk, NY, USA). Each included patient contributed with one target implant and was, therefore, considered as the statistical unit. Descriptive statistics were also performed for recorded clinical parameters (i.e., PI, BOP, PD and MR). Dummy regression was performed to assess association of mean rounded BOP values with mean PD values. For each clinical parameter, values were compared at the patient level among the different time points (i.e., baseline and the follow-ups at 2 and 9 years) using the Friedmann test. In case of significance, the Wilcoxon signed rank test was utilized as post-hoc test. To assess differences in clinical parameters at 2 years between patients who dropped out before the 9-year follow-up and those who did not, a Mann–Whitney *U* test was utilized. Kruskal-Wallis test was used to assess differences in mean BOP at 9-year follow-up among patients treated for peri-implantitis, mucositis or who did not receive any treatment. A Mann–Whitney-*U* test was used to assess differences between patients who were treated for peri-implantitis and those who were not. The results were found significant at $p < .05$. The *p*-values were adjusted using the Bonferroni method.

TABLE 1 Patient demographics and implant site characteristics after 2- and 9-year follow-up

Variables	2-year follow-up	9-year follow-up
Patient number (*n*)	48	30
Female	31	19
Male	17	11
Age (years at implant placement)	47.6 ± 13.4	49 ± 12.8
Observation period (months)	25.5 ± 5.8	111.1 ± 2.2
Patient with multiple implant sites	15	10
Patients with 1/2/3 implants	33/10/5	10/6/4
Patients treated by surgeon 1/2/3	7/29/12	7/16/7
Target implant sites	48	30
Location maxilla	13	10
Location mandible	35	20
Target implant sites with augmentation	19	11
Simultaneous grafting of a dehiscence-type defect	12	7
Internal sinus floor elevation	6	3
External sinus floor elevation	1	1

Note: Data are presented as frequency or as mean ± SD.

3 | RESULTS

Thirty patients out of the 46 eligible ones were available for the 9-year follow-up assessment. All the patients responding to the 9-year follow-up recall met the inclusion criteria. Demographic data and implant site characteristics are summarized in Table 1. Among the 16 patients lost to the 9-year follow-up, one patient moved to another state, another one unfortunately died, while the remaining 14 patients were not reachable. For all the investigated clinical variables, there was no significant difference at two years between the

subjects that reached the 9 years follow-up and the group of subjects that dropped out after the 2 years of follow-up (i.e., PI, $p = .565$; BOP, $p = .506$; PD, $p = .639$; MR, $p = .548$). Between 2 and 9 years, all the included patients were under regular professional maintenance regimen either at the Department (10%) or at the referring dentist (90%). The mean follow-up period was 111.1 ± 2.2 months from the time of implant placement. Among the included patients, one target implant 5 mm in diameter and 11 mm in length positioned in the lower molar in a female patient failed after 110 months from implant placement (Figure 2). Therefore, data recorded from the remaining 29 target implants were included in the statistical analysis.

3.1 | Clinical measurements and biological complications

The clinical parameters (i.e., PI, BOP, PD and MR) at patient level at different time points (i.e., baseline and the follow-ups at 2 and 9 years) are reported in Table 2 and Figure 3. The *p*-values adjusted using Bonferroni method are presented in Table 3 for all the investigated *post-hoc* comparisons, if the Friedman test was significant. The Friedmann test failed to find any significant difference among BOP ($p = .555$) and MR ($p = .077$) values; therefore, *post-hoc* comparison was not performed for these clinical parameters.

The majority of the patients (82.8%) presented no plaque around the target implants at the baseline. Mean PI values obtained in the early phase increased over time. Mean PI values at both 2 and 9 years were significantly higher compared to those recorded at baseline. Although the descriptive analysis indicates an increase in PI between 2 and 9 years (Figure 3a), no statistically significant difference was detected.

At 9-year follow-up, 16 (55%) out of 29 target implants included for the analysis presented a BOP of 0%. A maximum of two bleeding sites was detected in all the remaining cases, except for two target implants presenting BOP+ in 3 out of 6 sites. No significant differences in mean BOP values were evidenced between the three time points (Figure 3b). Before the 2-year follow-up, among the included 29 target implants, 10 implants diagnosed with peri-implant mucositis received mechanical debridement and local antiseptic therapy with chlorhexidine digluconate. Whilst, 10 implants diagnosed with peri-implantitis were treated with Er:YAG laser therapy, as described elsewhere (Schwarz et al., 2015). Kruskal-Wallis test revealed no significant differences in mean BOP at 9 years between the implants previously treated for peri-implantitis, the ones treated for peri-implant mucositis and the remaining 9 implants ($p = .456$). Similarly, no differences were observed between the group treated for peri-implantitis and all the others ($p = .845$).

The highest PD value registered at 9-year follow-up was of 6 mm in two patients, which was recorded in only one site per target implant. In these patients, the x-ray confirmed a bone level <3 mm. According to the given definition (Berglundh et al., 2018), no peri-implantitis was diagnosed. However, at 9 years signs of inflammation (i.e., BOP+) at the target implant were observed in 13 out of 29 patients with survived target implants (44.8%).

As shown in Figure 3c, an increase in mean PD values was observed during the first two years after loading, whereas the values remained constant from 2- to 9-year follow-up. Significant differences in mean PD values were found between the baseline and both 2 and 9 years. The worst PD value per time point at each target implant is reported in Figure 4, showing similar outcomes at 2 and 9 years.

A graphical overview of the correlation at 9-year follow-up of the site-specific PD values and the concomitant presence or absence of BOP at the same sites is provided in Figure 5. Furthermore, dummy regression revealed that mean rounded BOP values of 50%, which was the highest value reported at 9-year follow-up and occurred just in two patients, were significantly associated with an increase of 0.94 mm in PD values.

At 9-year follow-up, the mean MR values were below 1 mm for all the included target implants (Figure 3d). A recession of 1 mm at least at one site was recorded around only 10 out of 29 target implants. Among these, only one patient presented an exposure of 2 mm of the transgingival portion of the implant, specifically on the lingual aspect. Details on worst MR values are reported in Figure 6. No significant differences in MR values could be detected between different time points, confirming the stability of the results overtime.

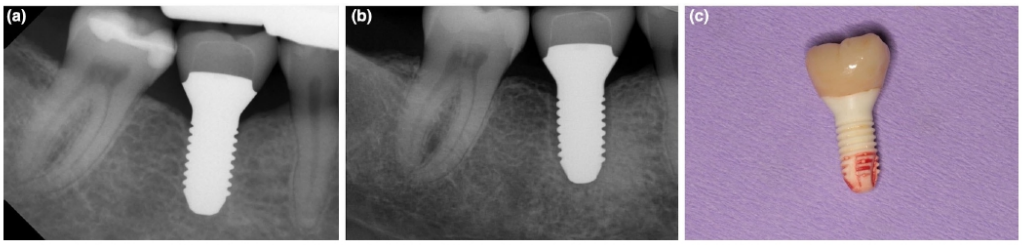

FIGURE 2 Case of implant failure between 2- and 9-year follow-up. (a) Intraoral radiograph at 6 months after crown fitting confirming implant osseointegrarion; (b) intraoral radiograph at 110 months after implant placement showing the characteristic peri-implant radiolucency; (c) removed implant. The absence of an adequate contact point after the replacement of the restoration at tooth 37 might have played a role in implant failure.

Overall, clinically, an improvement of soft-tissue conditions was observed. A representative case of creeping attachment leading to a full coverage of the initial buccal mucosal recession at the target implant (46) and at the two neighbouring ceramic implants is shown in Figure 7.

3.2 | Mechanical and technical complications

Between the 2-year and 9-year follow-up, three technical complications occurred in two patients (6.9% at patient level). These included one abutment decementation and one case of crown fracture followed by the loosening of the new crown. These complications were observed after a mean time of 43.7 months (SD 36.6) from the initial loading or from the new crown fitting.

TABLE 2 Clinical parameters (mean and SD) at the target implant, that is baseline and the follow-ups at 2 and 9 years

	Baseline		24 months		9 years	
Index	Mean	SD	Mean	SD	Mean	SD
PI	0.09	0.26	0.26	0.27	0.33	0.28
BOP (%)	22.4	29.4	14.7	17.1	12.9	15.8
PD (mm)	1.9	0.8	3.2	0.5	3.0	0.6
MR (mm)	0.2	0.4	0.1	0.1	0.1	0.2

Note: $n = 29$ target implants within the 30 patients included in the current study (1 implant failed).

Six mechanical complications, consisting in the fracture of the fibreglass abutment, were registered in six patients (20.7%). One of those was detected in the patient who had previously experienced two complications at crown level. Mechanical complications were successfully resolved with the removal of the fractured abutment and the delivery of a new crown. Mechanical complications occurred after a mean observation time of 53.7 months (SD 22.9) from the initial loading or from the new crown fitting.

4 | DISCUSSION

Thirty patients with one target implant each responded to the 9-year recall invitation. Among them, only one implant was lost. Albeit, no case of peri-implantitis was diagnosed. Mean PI values tended to increase between 2- and 9-year follow-up, while mean BOP and PD values remained stable over the same observation time. Approximately two third of the implants included in the analysis exhibited no mucosal recession (19 out of 29 target implants) and all the remaining implants but one presented a maximum MR value of 1 mm, confirming the healthy conditions of the peri-implant soft tissues. Contrary to our previous examination, a high rate of technical and mechanical complications was registered. Nevertheless, they were all resolved with the replacement of the prosthetic components and none of them affected the integrity of the implants.

As emerges from a systematic review evaluating the clinical performances of zirconia implants (Roehling et al., 2018), the broad

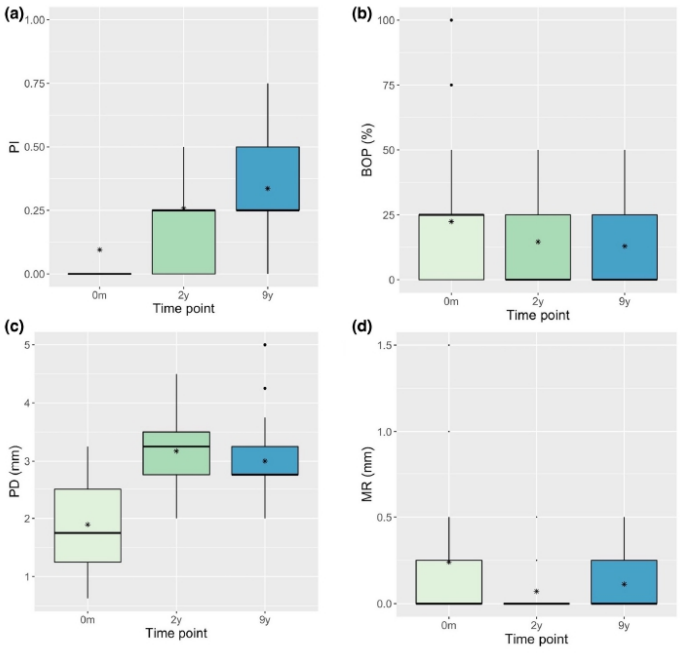

FIGURE 3 Boxplot representations at different time points (i.e., baseline and the follow-ups at 2 and 9 years) of PI (a), BOP (b), PD (c) and MR (d) recorded at the 29 target implants considered for analysis at the 9-year follow-up. Mean value is reported as follows (⁎).

TABLE 3 Clinical parameters

Grouping variable	Comparator 1	Comparator 2	*p*-value
PI	Baseline	2 years	.026*
	Baseline	9 years	.001**
	2 years	9 years	.881
PD	Baseline	2 years	.000***
	Baseline	9 years	.001**
	2 years	9 years	.345

Note: The Friedmann test was performed for each investigated clinical parameter (i.e., PI, BOP, PD and MR) to compare the values at the patient level among the different time points (i.e., baseline and the follow-ups at 2 and 9 years). In case of significance, a post-hoc Wilcoxon signed rank test with Bonferroni *p*-value adjustment was utilized. The adjusted *p*-values from the post-hoc test are reported.

*$p < .05$; **$p < .01$; ***$p < .001$.

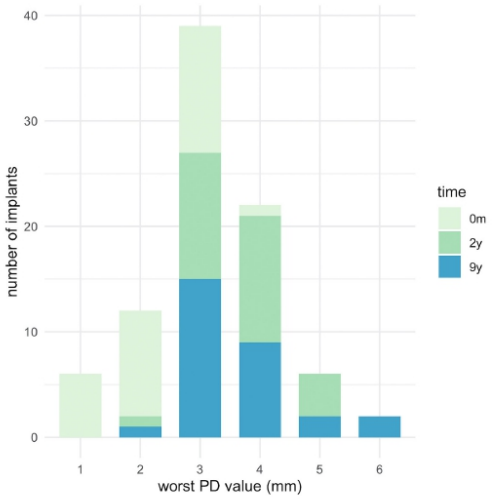

FIGURE 4 Bar chart reporting worst PD value at each target implant at different time points (i.e., baseline and the follow-ups at 2 and 9 years).

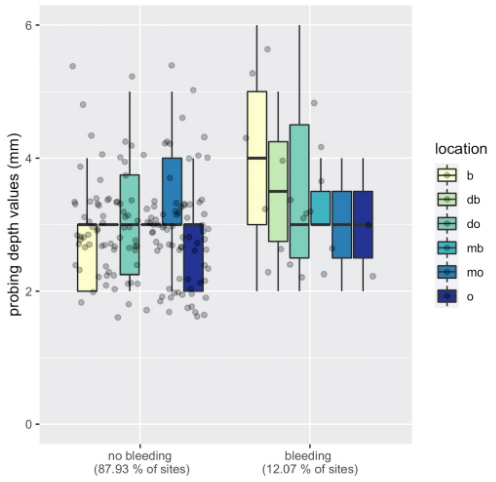

FIGURE 5 Boxplot illustrating site-specific PD values (i.e., b, db, do, mb, mo, o) recorded at the 29 target implants at 9-year follow-up based on the concomitant presence or absence of site-specific BOP.

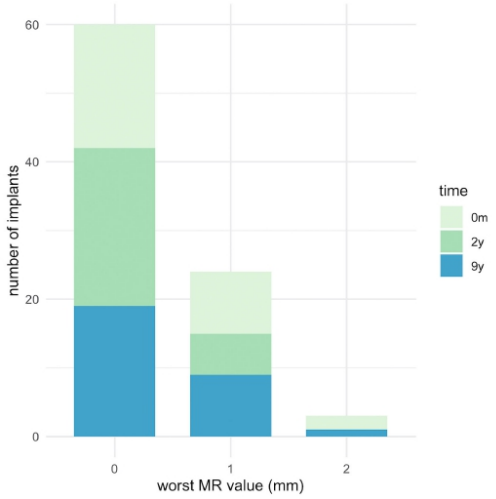

FIGURE 6 Bar chart reporting worst MR value at each target implant at different time points (i.e., baseline and the follow-ups at 2 and 9 years).

majority of the included studies were conducted on one-piece zirconia implants, and only 4 out of 18 on two-piece implants (Becker et al., 2017; Brüll et al., 2014; Cionca et al., 2015; Payer et al., 2015). Interestingly, only two studies investigated commercially available implants (Becker et al., 2017; Brüll et al., 2014). The first one consisted in the previous study of our group (Becker et al., 2017), in which two-piece zirconia implants restored with fibreglass abutments and all-ceramic single crowns revealed a high survival rate of 95.8% at a mean survival time of 32.9 months. The data were in line with results obtained in the other study utilizing the same commercially available implant system, reporting on an overall survival rate of approximately 96% after 3 years (Brüll et al., 2014). However, it has to be noted that both two-piece and one-piece implants were included in that retrospective analysis. Moreover, implants were provided either with single- or multi-unit fixed restorations and outcomes where not stratified for implant and prosthesis type. The implant loss documented in the current study has to be added to

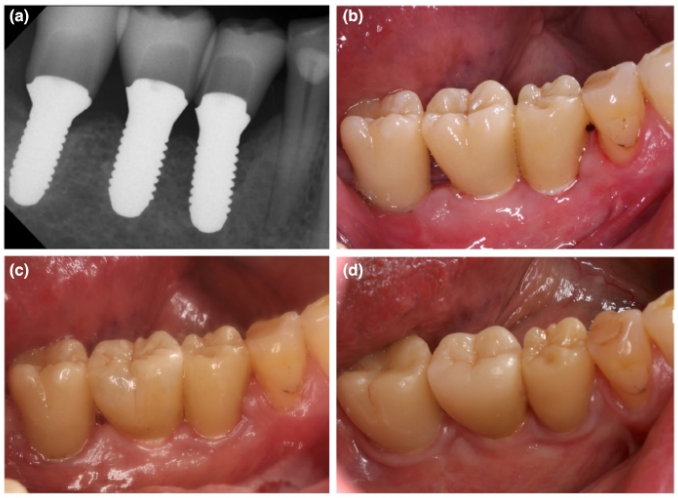

FIGURE 7 Representative case of long-term follow-up patient. (a) Intraoral radiograph and (b) clinical photo taken at crown fitting. Clinical images 2 years (c) and 9 years (d) after implantation, confirming the improvement and the long-term stability of peri-implant soft-tissue health.

the two previously reported failures (Becker et al., 2017). However, the pool of patients here included represents only a subgroup of the original group of participants, hence no cumulative survival rate can be calculated.

Plaque was detected around the majority of the target implants (22 out of 29). Despite no significant difference in mean PI was detected between 2- and 9-years, values tended to increase over-time. By contrast, in Koller et al. PI values significantly decreased between 30 and 80 months of loading of two-piece zirconia implants supporting single-unit crowns (Koller et al., 2020). Adequate daily at-home implant care as well as regular attendance to maintenance recall programs are considered fundamental for the long-term success of implant treatments (Brunello et al., 2020; Heitz-Mayfield & Mombelli, 2014; Roccuzzo et al., 2010; Schwarz et al., 2021). The decision to follow supportive care programs outside the clinic at the referring dentist was left to the patients after two years of follow-up. However, despite the impact of the quality and frequency of supportive maintenance care provided could not be assessed, since the plaque scores were relatively low at the final visit, the maintenance protocols are likely not to have confounded the results.

As regards mean BOP values, in our previous investigation they significantly increased over the first 12 months, while a significant decrease was found at 24 months (Becker et al., 2017). The favourable outcome was ascribed to the effective non-surgical treatments performed between the two time points for the management of peri-implant diseases (Schwarz et al., 2015). Thereafter, BOP at the available target implants remained almost unvaried, with mean values of 14.7% (SD 17.1) and 12.9% (SD 15.8) at 2 and 9 years of follow-up, respectively. Interestingly, no statistical difference was detected in mean BOP values at 9 years between target implants previously treated for peri-implantitis with laser and the remaining implants. The opposite trend was encountered in the prospective study of Koller et al. (2020), where zirconia implants were associated with a significantly higher BOP score at 80 than at 30 months from crown fitting, with mean BOP value of 16.43% (SD 6.16) at the latest time point. Whereas, six years after loading, the modified Sulcus Bleeding Index (mBI) (Mombelli et al., 1987) values at the surviving implants were equal to 28.5% and 3% for mBI>0 and mBI>1, respectively (Cionca et al., 2021).

Among the 30 included implants, one failed. Localized PD values of 6 mm were detected only in two patients in a singular point per target implant. However, this clinical observation was not accompanied by interproximal bone loss as compared to the time of crown fitting. A higher number of sites with PD values higher than 5 mm was documented in another prospective study on two-piece zirconia implants, reaching 7.5% of sites (17 out of 222) at 6 years after loading (Cionca et al., 2021). It has to be noted that in the current study the mean PD values were found to set around 3 mm after two years of follow-up and subsequently remained constant.

As regards soft-tissue healing, median MR values of 0 mm at all time points and localized MR of maximum 1 mm (except for a 2 mm recession) in approximately 35% of the target implants included at the 9-year examination were recorded. The present data supports previous findings observed both in pre-clinical and clinical studies (Becker et al., 2017; Kohal et al., 2004; Lee et al., 2019; Liñares et al., 2016; Welander et al., 2008).

In our short-term evaluation (Becker et al., 2017), only one mechanical complication was registered, consisting in the fracture of the fibreglass abutment in a patient that did not attend the 9-year recall visit. Among the subgroup of target implants here included, the majority of the complications occurred at the abutment level. In details, the abutment was found decemented in one case, whilst the fracture of the fibreglass abutment was observed six times. Although it can be hardly proven in vivo, in some cases abutment fractures might chronologically follow their loosening. Hence, it can be speculated that the correct cementation of the abutment represents a critical

step for the long-term success of the restorations. In a retrospective study utilizing the same implant system (Brüll et al., 2014), no loss of abutment retention or integrity was reported over an observation time up to 3 years.

In other studies, zirconia abutments were connected by adhesive luting to the zirconia implants, to support cemented single-unit all-ceramic restorations (Cionca et al., 2015, 2021; Koller et al., 2020; Payer et al., 2015). In the prospective study of Cionca et al., only two abutment-related complications were reported in the short term (Cionca et al., 2015). Nevertheless, at the 6-year follow-up evaluation numerous mechanical and technical complications were registered among the 24 included patients with a total of 39 implants, in particular 6 abutment fractures and 6 cases of loss of retention at the abutment-crown complexes. In a randomized clinical trial, aside from the failed implants (2 out of 16 in the zirconia group), any mechanical or technical were reported. However, the authors emphasised the challenges related to the cementation of the abutment (Koller et al., 2020).

As this phase is deemed to be highly sensitive, it would be interesting to investigate if there is any correlation between the experience of the prosthodontist and the final outcomes. Similarly, the morphology of the abutment, the abutment material, the type of cement, the cementation technique (e.g. use of the rubber dam), as well as the implant design (i.e., bone level or tissue level) might have an effect on the abutment-implant connection.

Active matrix-metalloproteinase-8 (aMMP-8) in the peri-implant crevicular fluid (PICF) is considered an important biomarker for the onset and progression of peri-implant diseases (Ghassib et al., 2019; Ramseier et al., 2016; Wohlfahrt et al., 2014). The authors recognize the importance of assessing aMM8 levels in the PICF for research purposes. However, contrary to our previous investigation, it was decided not to collect PICF samples at the 9-year follow-up visit, because its quantification would have not modified the treatment of peri-implant diseases if detected by means of clinical and radiological examinations.

Study limitations included the relative high rate of dropouts. Nonetheless, the reason why the patients were lost to follow-up was reported and statistical analyses accounted for them (Tonetti & Palmer, 2012). Further, when data are missing not at random (i.e. dropouts are related to unobserved information or to outcome variables) they could lead to considerable bias in the results (Fewtrell et al., 2008; Kristman et al., 2004; Touloumi et al., 2002). However, there was no significant difference after 2 years of follow-up in terms of clinical variables considered (i.e., PI, BOP, PD and MR) between the participants that reached the final investigation and the 16 dropouts. Therefore, the cohort of patients included at 9 years should truthfully represent the original one in terms of compliance and clinical conditions. Other limitations of the present study include the absence of a control group and the retrospective design of the study and the lack of longitudinal assessment of interproximal radiographic bone level, due to the strict compliance with the current national legislation. As clinical parameters (BOP and PD) can be considered predictors of disease progression (Berglundh et al., 2021; Carcuac et al., 2017; Karlsson et al., 2019), the sole presence of BOP+ in absence of PD values ≥6 mm was not considered sufficient for taking x-rays. Indeed, in these circumstances the therapeutic approach would have been in the first place non-surgical no matter what.

Furthermore, it is worth noting that nowadays zirconia implants are mainly used in the front areas for aesthetic purposes; however, this material might represent a valid alternative to titanium implants also in the posterior jaws. Hence, on one side our study design with implants exclusively positioned in the posterior areas might be considered as a limitation, on the other side this makes it particularly suitable to evaluate the behaviour of two-piece zirconia implants when subjected to higher loading.

In recent studies utilizing either one- or two-piece zirconia implants, participants generally reported good satisfaction (Cionca et al., 2021; Kohal et al., 2020). This aspect could be further investigated in future studies, to longitudinally assess patients' satisfaction about the treatment and related effects on their quality of life.

Finally, it has been demonstrated that the type of abutment substrate (i.e., titanium vs. zirconia) could have a relevant impact on the microbial adhesion and colonization (de Freitas et al., 2021; de Oliveira Silva et al., 2020). It would be interesting to characterize changes overtime in individual microbiological profile associated to two-piece zirconia implants restored with cemented fibreglass abutments and all-ceramic crowns. The impact of microbiota on the clinical outcomes could also be assessed.

In conclusions, within the limitations of the present retrospective cohort study, an overall stability of the results was registered between 2 and 9 years of follow-up. Two-piece zirconia implants supporting single-unit crowns could represent a valid solution for the rehabilitation of the posterior edentulous jaws. Despite the occurrence of several mechanical and technical complications, they were all successfully solved by replacing the prosthetic components.

AUTHOR CONTRIBUTIONS

Giulia Brunello: Data curation (equal); formal analysis (supporting); writing – original draft (lead); writing – review and editing (equal). **Nicole Rauch**: Data curation (equal); investigation (equal); writing – original draft (supporting); writing – review and editing (equal). **Kathrin Becker**: Formal analysis (lead); writing – original draft (supporting); writing – review and editing (equal). **Ahmad Hakimi**: Investigation (equal); writing – review and editing (equal). **Frank Schwarz**: Supervision (equal); writing – review and editing (equal). **Jürgen Becker**: Conceptualization (equal); project administration (equal); supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to this study.

DATA AVAILABILITY STATEMENT

Data will be provided upon reasonable request.

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