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ORIGINAL ARTICLE

Effects of different decontaminating solutions used for the treatment of peri-implantitis on the growth of *Porphyromonas gingivalis*-an *in vitro* study

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ABSTRACT

Implants have been considered the treatment of choice to replace missing teeth, unfortunately, peri-implant disease is still an unresolved issue. Contaminated implants may be decontaminated by physical debridement and chemical disinfectants; however, there is a lack of consensus regarding the ideal techniques/agents to be used for the decontamination. The objective of our study was to compare the decontaminating efficacy of different chemical agents on a titanium surface contaminated with *Porphyromonas gingivalis*, a typical representative of the bacterial flora associated with peri-implantitis. Commercially pure Ti grade 4 discs with a polished surface were treated with a mouthwash containing chlorhexidine digluconate (0.1%), povidone-iodine (PVP-iodine) solution (10%) or citric acid monohydrate (40%). Qualitative and quantitative assessment of cellular growth and survival were assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and scanning electron microscopy (SEM). Significant differences in the quantity of *P. gingivalis* could be observed after 6 days of incubation. A numerical, but not statistically significant ($P = 0.066$) decrease in the amount of living bacteria was observed in the group treated with the PVP-iodine solution as compared to the control group. The chlorhexidine (CHX)-treated group presented with significantly higher cell counts, as compared to the PVP-iodine-treated group ($P = 0.032$), while this was not observed compared to the control group and citric acid-treated group. Our results have also been verified by SEM measurements. Our results suggest that for *P. gingivalis* contamination on a titanium surface *in vitro*, PVP-iodine is a superior decontaminant, compared to citric acid and chlorhexidine-digulconate solution.

KEYWORDS

implant, *Porphyromonas gingivalis*, oral surgery, peri-implantitis, titanium

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INTRODUCTION

Over the past decades, implants have been considered the treatment of choice to replace missing teeth, as they have shown high survival rates (ranging from 90 to 98%) after being in function for at least 10 years [1–6]. Unfortunately, subsequent development of peri-implant diseases is still an unresolved issue [7–10]. Peri-implant infections include peri-implant mucositis (which can be defined as a reversible inflammatory response of the peri-implant soft tissues without bone loss) and peri-implantitis (an inflammatory process resulting in the loss of supporting bone associated with suppuration and bleeding) [11–14]. Marginal peri-implantitis can be traced back to infectious and biomechanical factors [15]. One of the key events in the pathogenesis is the development of microbial colonization on the surface of the dental implant [16, 17]. Peri-implant infections have mostly been linked to Gram-negative anaerobic bacteria [18, 19]; the toxin release by these pathogens provokes a massive immune response, which causes bone degradation, ultimately leading to implant loss [20].

Clinical signs of peri-implantitis include increased probing depths, mucosal recession, fistula formation, suppuration, mucosal swelling and bleeding on probing (BOP) [13, 21, 22]. Once peri-implant disease is diagnosed, the treating physician will be hard-pressed to deal with this issue. Various treatment modalities have been suggested and attempted with the aim of treating this oral pathology. In the case of peri-implant mucositis, non-surgical mechanical treatment may be effective and the outcome of therapy may be further enhanced with the usie of antimicrobial mouth rinses [11]. However, for peri-implantitis lesions, nonsurgical therapy alone was found to be ineffective [23]; for successful surgical interventions, the contaminated implant surface must be entirely decontaminated. However, there is a lack of consensus regarding the techniques/agents to be used for decontamination [11]. Several agents have been suggested by the literature; nevertheless, citric acid, chlorhexidine (CHX) and povidone-iodine (PVP-iodine) are probably the most often discussed ones [24, 25].

The objective of our study was to compare the decontaminating efficacy of the abovementioned three chemical agents on polished titanium (Ti) surfaces infected with *Porphyromonas gingivalis*, a typical representative of the bacterial flora associated with peri-implantitis.

MATERIALS AND METHODS

Preparation of Ti implant discs, sample design

During the present study, mechanically polished Ti implant discs (9 mm in diameter and 2 mm in thickness; commercially pure (CP) Grade 4; Protetim Ltd., Hódmezővásárhely, Hungary) were used. Similarly to the transgingival part of dental implants, the discs were polished to a surface roughness not exceeding 0.2 μm [10]. After cleaning in an ultrasonic bath with acetone, absolute ethanol and distilled water for 15 min, each sample was dried before use. The Ti discs were grouped into four different treatment groups, each group consisting of $n = 4$ four pieces for each independent experiment. The *first* group was the control group: after the cleaning and drying of the discs, no chemical treatment was applied. The others were immersed into a solution of one of the dental implant surface decontaminating agents for 60 min, the following chemical agents were used in this study: 0.1% chlorhexidine digluconate (*second* group; Corsodyl®, GlaxoSmithKline, Brentford. UK), 10% PVP-iodine solution (*third* group; Betadine®, Purdue Pharma LP, Stamford, CT, USA) and 40 w/w% citric acid monohydrate solution prepared in deionized water (*fourth* group; Thermo Fisher Scientific, Hampton, VI, USA). The discs were always handled with Ti forceps to avoid contamination of the Ti surface with other metals [18]. For each round of experiments, $n = 16$ Ti samples were used: $n = 12$ Ti discs were immersed into one of the prophylactic agents, and $n = 4$ samples were left untreated (control group) but went through the same cleaning procedure. Three independent experiments were performed, with altogether $n = 48$ titanium discs. After 60 min of immersion, the samples were washed with ultrapure water (Thermo Fisher Scientific, Hampton, VI, USA) and dried.

The application time of different decontamination solutions corresponds to different time periods or effects of accumulated use under clinical circumstances: for CHX, the dose corresponds to 4 months of regular use, for citric acid, it corresponds to 12 clinical uses, while for PVP-iodine treatment, the dose was equivalent for 20 clinical uses. This was calculated based on available literature data and the manufacturer's instructions [26–28]. All treated and control discs were steam-sterilized at 160 C for 45 min and stored in closed packaging until use. The experiments were always performed within packaging expiration time, which was 14 days [19].

Preparation of *Porphyromonas gingivalis* inoculum

Columbia agar base (Oxoid, Basingstoke, United Kingdom) supplemented with 5 v/v% cattle blood, hemin and vitamin K_1 was used for the culturing of *P. gingivalis*. Fresh colonies of *P. gingivalis* strain ATCC 33277, incubated in an atmosphere of 90% N_2, 5% H_2 and 5% CO_2 in an anaerobic environment for 48 h (Concept 400 anaerobic incubator, Biotrace International Plc., UK) were suspended in reduced Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, United Kingdom) and used after gentle dispersion (at a bacterial cell density of McFarland 1.0 dilution). 2 mL aliquots of these bacterial suspensions were immediately plated onto 24-well sterile microtiter plates, containing the different Ti discs from the respective groups. Every second day, the bacterial suspension was changed for a fresh solution. After 6 days of anaerobic incubation (under 90% N_2, 5% H_2 and 5% CO_2 in the abovementioned anaerobic incubatior), the samples were removed from the bacterial culture.

MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was used for the determination of bacterial growth and viability, during which the activity of mitochondrial dehydrogenases in living bacterial cells is measured, corresponding to a color change (due to the reduction of yellow MTT into purple, water-insoluble tetrazolium salt), which may be quantifiable with spectrophotometric methods [29]. Cells were seeded into 48-well culture plates at a density of 3,000 or 10^4 cells/well and grown on Ti discs in culture media for 24 or 72 h, respectively. The supernatant was removed and replaced with 0.5 mg/mL MTT solution (Sigma-Aldrich GmbH, Germany) in RPMI medium without phenol red. After incubation for 4 h at 37 °C, the medium was gently removed from each well and the crystallized dye was solubilized with 2% sodium dodecyl sulfate (SDS) and 0.04 mM HCl in absolute isopropanol [30]. The optical density of was determined at 540/630nm with a Multiscan EX spectrophotometer (Thermo Labsystems, Vantaa, Finland) and Ascent Software (Thermo Labsystems, Vantaa, Finland).

Scanning Electron Microscopy (SEM) studies

After treatment with the disinfectant solutions and bacterial incubation, the Ti discs were treated with the following method for fixation: dehydration of the surface bacteria and bacterial biofilm, first by rinsing with ethanol solutions of increasing concentrations, (30–50–70–100 V/V%) then by a mixture of ethanol and acetone (90–10, 70–30, 50–50, 30–70, 10–90, 100% of acetone). Critical point drying (determined by an SPI 1320 apparatus) was applied, after which the discs were gold-coated by means of an Edwards sputter coater and subjected to scanning electron microscopy (SEM) with JEOL JSM-7100F/LV instrument.

Statistical analysis

The results of the measurements were collected in a spreadsheet file Microsoft Excel 2013 (v15.0) (Microsoft Corporation, Redmond, WA, USA). Statistical analysis was performed using Statistica for Windows 10.0 (Statsoft, Tulsa, OK, USA). Besides the descriptive statistics, groups defined by the treatment of the chemical agents were compared with one-way ANOVA (with Tukey post hoc analysis). P values <0.05 were considered statistically significant.

RESULTS

Assessment of biofilm-formation by SEM studies

Various amounts of biofilm evolved on the Ti disc surfaces depending on the treatment applied (see groups 1–4). This is well illustrated by the SEM images taken at day 6. Fig. 1 shows the surface of a control disc after 6 days of incubation with *P. gingivalis*. Some of the bacteria occurred in multiple layers, in an interconnected manner, in addition a biofilm has noticeably formed. Fig. 2 shows the surface of a disc treated with chlorhexidine and incubated with *P. gingivalis* for 6 days. Bacterial biofilm could be observed in several layers on the surface, which is not continuous. SEM analysis shows no substantial difference in comparison with the control group. Fig. 3 shows a SEM image of the

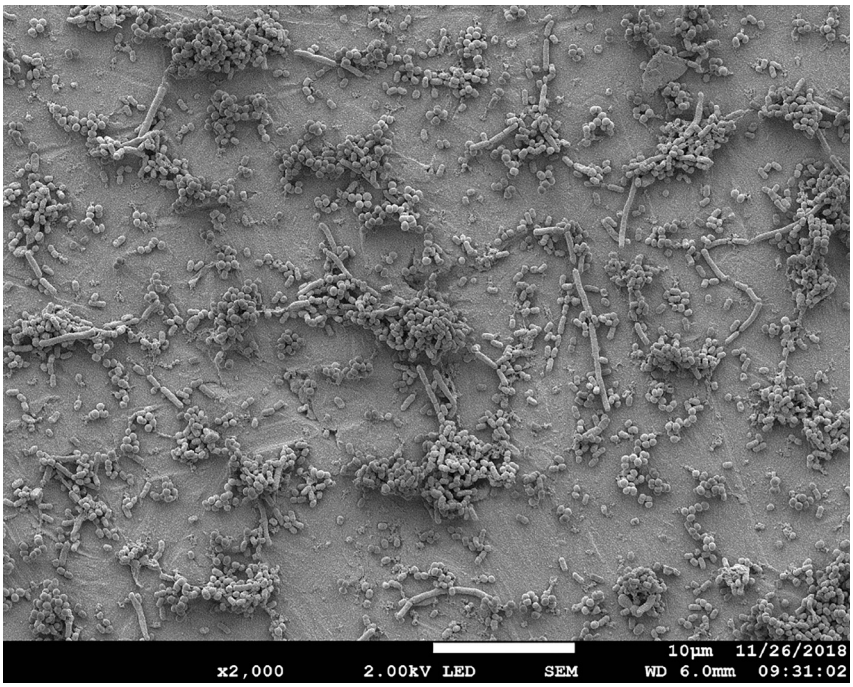

Fig. 1. Scanning electron microscope (SEM) image of an untreated control disc. Magnification: 2,000×

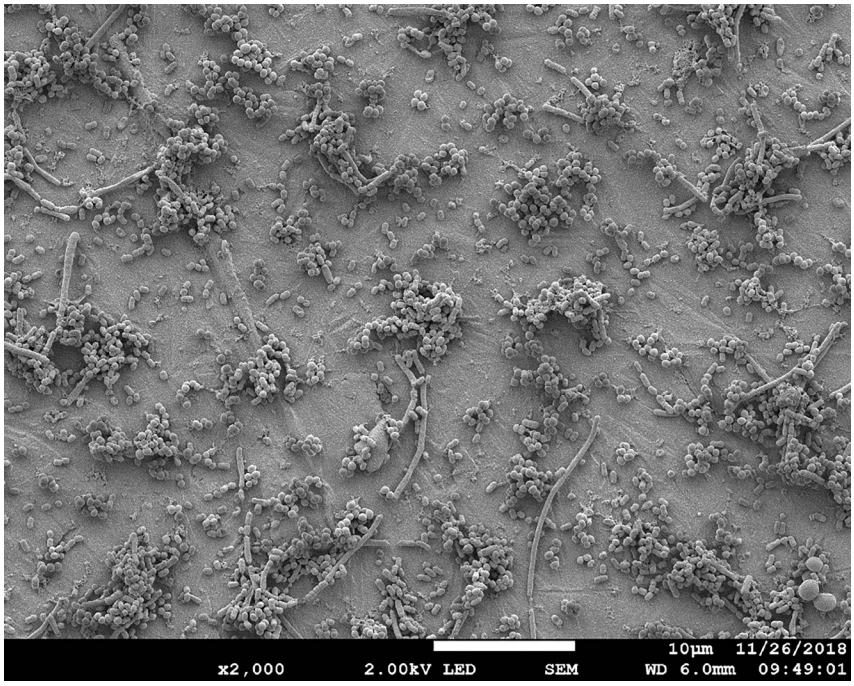

Fig. 2. Scanning electron microscope (SEM) image of a Ti disc treated with chlorhexidine and incubated with *Porphyromonas gingivalis* for 6 days. Bacterial growth is readily observable. Magnification: 2,000×

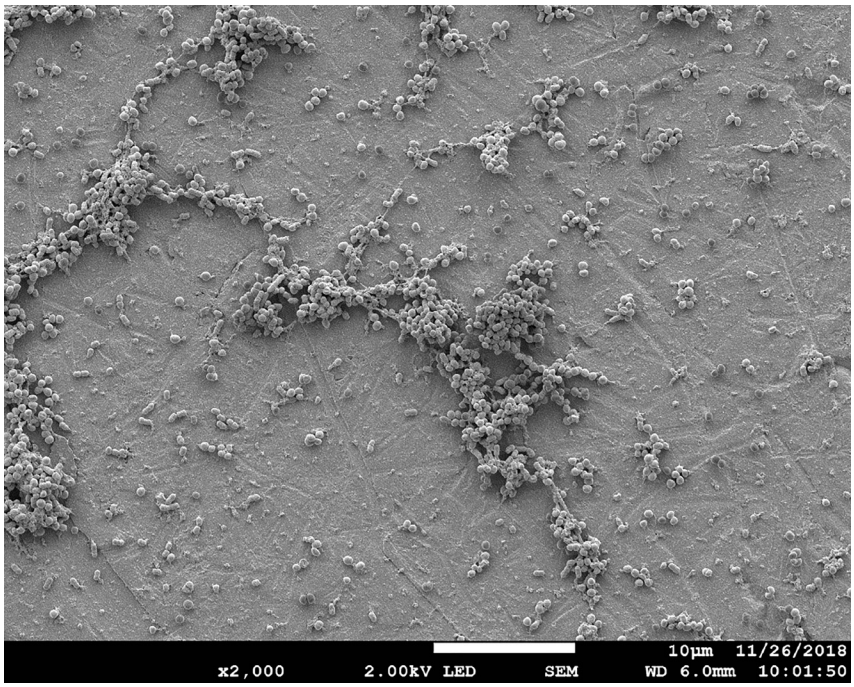

Fig. 3. Scanning electron microscope (SEM) image of a Ti disc treated with citric acid monohydrate and incubated with *Porphyromonas gingivalis* for 6 days. Bacterial growth is observable in multiple layers. Magnification: 2,000×

surfaces of a Ti disc after treatment with citric acid-monohydrate and incubation with the model pathogen: some bacteria formed multiple layers of biofilm. Fig. 4 shows a Ti surface after treatment with 10% solution of PVP-iodine: note that the bacterial growth is negligible as compared to the rest of the treatments. Active reproduction of the bacteria was observed and the formation of a monolayer has not yet started.

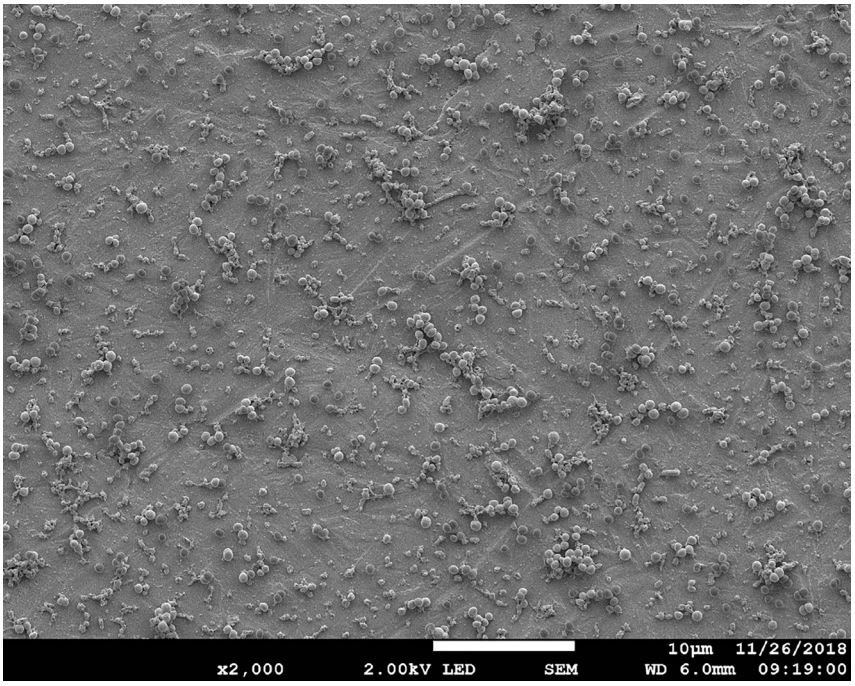

Fig. 4. Scanning electron microscope (SEM) image of a Ti disc treated with povidone-iodine and incubated with *Porphyromonas gingivalis* for 6 days. Bacteria are present, but to a notably lesser extent than on the citric acid- and CHX-treated and control surfaces. Magnification: 2,000×

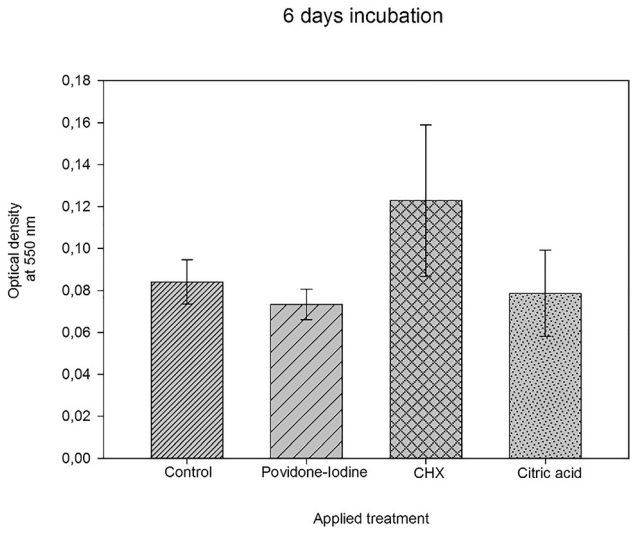

Fig. 5. Bacterial cell viability on the surface of Ti discs after 6 days of incubation with *Porphyromonas gingivalis*

Assessment of the bacterial cell viability after different treatments

Significant differences in the quantity of *P. gingivalis* could be observed after 6 days of incubation. A numerical, but not statistically significant ($P = 0.066$) decrease in the amount of living bacteria was observed in the group treated with the PVP-iodine solution as compared to the control group. The CHX-treated group presented with significantly higher cell counts, as compared to the PVP-iodine-treated group ($P = 0.032$), while this was not observed compared to the control group and citric acid-treated group ($P > 0.05$; Fig. 5). Results by measurement are presented in Table 1.

DISCUSSION

During our present study, the effects of different disinfectants were assessed on bacterial cell viability on Ti discs, where *P. gingivalis* was chosen as a model microorganism. The results obtained during our experiments confirmed our hypothesis that the use of different chemical agents has a substantial influence on bacterial growth on the dental material. Our *in vitro* results suggest that the right choice of disinfectant influences the resistance of Ti implant surfaces to bacterial growth, which should be considered a clinically important finding. In addition, chemical disinfection alone was found to be ineffective (no statistically significance was shown), which, however, is in accordance with the literature.

Considering both the quantitative (MTT assay) and qualitative (SEM studies) results of this study, the most effective treatment modality in this study was PVP-iodine. Several studies in the literature have reported on the highly substantive nature of CHX on titanium surfaces [31]; in their study, Kotsakis et al. concluded that the use of CHX is not recommended for the decontamination of titanium surfaces [32]. In contrast to CHX, exposition of osteoblast cell lines to PVP-iodine has been reported to lead to an initial decrease, but an increase of the mineralization activity over a longer period of time [33]. Furthermore, PVP-iodine has a broad antibacterial spectrum, including bacteria that

Table 1. Results of individual measurements of the MTT assay

	Treatment groups			
$OD_{550/630nm}$	Control	Povidone-iodine	CHX	Citric acid
Measurement 1	0.093	0.072	0.196	0.108
Measurement 2	0.109	0.085	0.169	0.133
Measurement 3	0.114	0.093	0.240	0.125
Measurement 4	0.083	0.070	0.094	0.043
Measurement 5	0.076	0.064	0.064	0.044
Measurement 6	0.066	0.090	0.076	0.043
Measurement 7	0.084	0.061	0.093	0.071
Measurement 8	0.069	0.066	0.089	0.062
Measurement 9	0.063	0.060	0.085	0.079
Average	*0.084*	*0.073*	*0.123*	*0.079*
SD	*0.018*	*0.013*	*0.06*	*0.04*

have been associated with periodontal and peri-implant microflora [34]. The idea of using citric acid to treat peri-implantitis was based on empirical clinical success with its use in treating periodontitis, rather than results of systematic scientific research [35]. This positive effect of citric acid has been widely observed in periodontitis, revealing that this chemical agent can stimulate cementogenesis and may also enhance reattachment to the root surface [36–39]. In addition, citric acid has been shown to inhibit the growth of bacteria on root surfaces affected by periodontal diseases [40]. Although citric acid has shown great capacity for implant surface decontamination, more studies are needed to test and verify its efficacy [5].

The use of chlorhexidine gluconate (CHX) is well documented in periodontal therapy and we included this agent in our studies due to its widespread use; its use has been proven to be effective in reducing periodontal inflammation and in controlling subgingival plaque formation [41]. This agent is thought to be a non-specific antibacterial agent and it directly interferes with the cell walls of bacteria, resulting in cell lysis [42, 43]. The use of CHX is advantageous due to its substantivity, which allows the agent to be absorbed into hard and soft oral tissues and be released over time. This effect can last up to 12 h. Nonetheless, several disadvantages are related to its use, like taste alterations, staining, and slight increase in calculus formation [43]. CHX have been widely used to treat peri-implantitis, but no study so far have managed to demonstrate the superiority of chlorhexidine over other decontamination agents [5]. In several previous studies on non-surgical peri-implantitis therapy, CHX was preferentially used as an antiseptic adjuvant, with variable clinical success [44–48]. Patients received a one-stage full-mouth scaling with or without chlorhexidine led to an improvement of the clinical parameters and a temporary reduction of the microflora at implants with mucositis [42]. More importantly, Kotsakis et al. have recently demonstrated that CHX is able to notably change the physicochemical properties of the titanium implant surfaces and markedly inhibit the activity of osteoblasts [32]. This raises the possibility of cytotoxicity, which questions the use of chlorhexidine in the treatment of peri-implant disease.

PVP-iodine is a topically used antiseptic agent with a broad antibacterial spectrum covering a multitude periodontopathogenic species. Lanker Klossner et al. [49] demonstrated that long-term use of PVP-iodine does not cause any bacterial resistance; however, Sahrmann et al. found that PVP-iodine reapplied frequently during scaling and root planning might enhance pocket depth reduction in initially deep pockets [34] and the adjunctive use of PVP-iodine during scaling and root planning may increase the clinical pocket depth reduction [35]. Another study showed the application of PVP-iodine gel in periodontal pockets allows a prolonged remnant effect compared with the solution PVP-iodine formula [36]. Although the efficiency of PVP-iodine seemed to be superior in comparison to citric acid based on the SEM findings, the statistical analyses did not show significant difference between these two agents.

During our studies, *P. gingivalis* was chosen as a model microorganism for antibacterial and biofilm-formation studies. The dental biofilm is a natural ecosystem, bacteria predominantly reside in structured, surface-attached communities embedded in a self-produced, extracellular matrix [24, 50, 51]. The oral Gram-negative obligate anaerobic bacterial species *P. gingivalis* is typically considered a so-called "late colonizer" of subgingival biofilms and has been related to several destructive periodontal diseases, including periodontitis and peri-implantitis [50]. The pathogenicity of *P. gingivalis* is reflected in a lot of different important virulence factors involved in tissue colonization and destruction, and interference with host defense systems. Peri-implant inflammation and lesions are of multifactorial nature, but it is a fact unequivocally supported by the literature that *P. gingivalis* plays a leading role in both cases [24, 50, 51]. In a previous study, it was demonstrated that *P. gingivalis* has the ability to colonize titanium surface in multiple interconnected layers, forming a biofilm, namely a polysaccharide coat (glycocalyx), which is a protective layer for the bacterial population [52, 53].

CONCLUSION

During our experiments, a comparison of different chemical disinfectant treatment protocols was performed with the aim of assessing the efficacy as antibacterial agents in clinical situations. The proliferation of *P. gingivalis* was measured, which is a pathogen, which plays a crucial role in the development of peri-implant inflammations. During the comparison we did find significant differences in microbial growth on the treated titanium discs, compared to the control group. However when compared to the chlorhexidine-digluconate-treated group, disk treated with PVP-iodine showed a marked reduction in bacterial count. Our results suggest that PVP-iodine is superior to CHX for the chemical disinfection of titanium surfaces in the context of *P. gingivalis* contamination. *P. gingivalis* still has a crucial role as a periodontopathogen, therefore studies regarding the response of this specific pathogen to various treatments does provide clinically useful knowledge to help combat periodontitis and peri-implantitis in the future.

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Conflicts of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

Data availability: The data used to support the findings of this study are included within the article.

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