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RESEARCH

Non-surgical treatment of residual periodontal pockets using sodium hypochlorite/amino acid gel and cross-linked hyaluronic acid—a 9-month pilot randomized controlled clinical trial

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Abstract

Objectives This pilot randomized controlled clinical trial compares the clinical outcome obtained in persistent periodontal pockets during 9-month follow-up of supportive periodontal step 4 treatment performed by either combining subgingival instrumentation with adjunctively used sodium hypochlorite/amino acid gel and crosslinked hyaluronic acid (xHyA) or subgingival instrumentation alone.

Materials and methods Study protocol is registered under NCT06438354 at Clinicaltrials.gov. Patients seeking further therapy after completed step 2 non-surgical periodontal treatment underwent either repeated subgingival instrumentation with adjunctive application of sodium hypochlorite/amino acid gel and crosslinked hyaluronic acid (group A) or repeated subgingival instrumentation alone (group B). One calibrated investigator performed the treatment sequence in both groups accordingly. Subgingival instrumentation of the residual pockets was carried out under local anaesthesia using hand- and ultrasonic instruments, as well as air polishing in both groups. Patients were instructed to continue oral hygiene without any restriction. At 3-month re-evaluation treatment was repeated accordingly at sites with persistent 5 mm probing depth and BoP+. Clinical attachment level (CAL), pocket probing depth (PPD), gingival recession (GR), and bleeding on probing (BoP) were recorded at baseline (T1), 3- (T2) and 9-month (T3) post-op, with CAL as a primary outcome measure.

Results In total 52 patients (20 females and 32 males, mean age 58.4 ± 2.4 years) presenting with 1448 sites which required further periodontal treatment were enrolled. Both groups exhibited homogeneity in terms of age, gender, smoking habit, initial number of sites, and BOP. At 9-month evaluation, PD reduction and CAL gain showed significant differences between the test and control group, favouring the adjunctive treatment. GR tended to exhibit more recovery in the test group compared to the control group. Although BOP frequency effectively reduced in both groups, there was no statistically significant difference between the two groups.

Conclusion Within the limits of the study, the present data indicates that, during subgingival instrumentation of persistent pockets, the adjunctive usage of sodium hypochlorite/amino acid gel and xHyA sufficiently improves the clinical outcomes. The continuous improvement of CAL in association with the GR scores observed in group A, indicates that sites subjected to adjunctive treatment may indicate a tendency for a regenerative response to treatment within the 9-month follow-up period.

Keywords Periodontitis · Cross-linked hyaluronic acid · Adjunctive treatment · Subgingival instrumentation · Sodium hypochlorite/amino acid · Persistent periodontal pockets · Non-surgical periodontal treatment

Introduction

Periodontitis, a predominantly chronic inflammatory disease caused by dysbiotic dental plaque biofilms, poses a substantial global health burden. According to the Global Burden of Disease Study, periodontitis ranks sixth worldwide in terms of prevalence, with an alarming 743 million reported cases [1]. Given the progressively aging global population and the increased retention of natural teeth, a

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heightened global burden is anticipated in the future [2]. Additionally, periodontitis demonstrates bidirectional links with systemic conditions such as atherosclerosis and diabetes [3, 4]. Consequently, the prevention, diagnosis, and effective treatment of periodontitis are paramount.

The primary goal of periodontitis therapy is to resolve periodontal inflammation, expressed by the absence of bleeding, and reduce probing depths to less than 4 mm, preferably alongside with minimal gingival recession [5, 6]. While the efficacy of non-surgical periodontal therapy is evident, residual periodontal pockets are regularly observed at re-evaluation performed during supportive periodontal therapy (SPT). Residual periodontal pockets act as reservoirs for pathogenic bacteria, which lead to the persistence in inflammation and correlate with an increased risk for tooth loss [7]. Since persisting inflammation exhibits a severe risk of disease progression, the timely treatment of respective periodontal pockets is a clinical imperative to establish long-term periodontal stability. Residual or persisting pockets are classified as such if sites present with 4 mm PPD and positive BOP or 5 and more mm in depth. According to the EFP treatment guidelines, these sites are supposed to refer to Step 3 therapy, which may encompass different surgical approaches or repeated instrumentation [8]. However, periodontal surgery may be technically challenging for general practitioners and associated with increased patient morbidity, albeit it remains the most effective treatment option according to the clinical literature in deeper sites [9]. Possible reasons for the inferiority of non-surgical re-instrumentation may include challenging removal of calculus due to restricted accessibility of sites with deep probing depth, or involvement of multi-rooted teeth [10, 11]. For this reason, various adjunctive protocols have been proposed to enhance the efficacy of non-surgical periodontal therapy recently. To date, many adjunctive interventions oscillate between antibacterial effect of local antibiotic chemotherapy, antiplaque chemical agents, or photodynamic therapy [12, 13]. While the use of biologics, such as enamel matrix derivatives, hyaluronic acid, and platelet-rich fibrin for regenerative periodontal surgery has steadily increased, applying these materials to non-surgical therapy is still uncommon [14–16]. Intriguingly, the integrative application of a sodium hypochlorite/amino acid and crosslinked hyaluronic acid (xHyA) as a regenerative adjunct in the non-surgical treatment of residual periodontal pockets has demonstrated significant attachment level gain in two retrospective studies [17, 18]. However, adequately controlled data supporting the efficacy of this protocol during supportive periodontal therapy is lacking.

This pilot randomized controlled clinical trial investigated the efficacy of adjunctive sodium hypochlorite, and xHyA in treating residual periodontal pockets during supportive periodontal therapy.

Materials and methods

Study design

The randomized controlled trial protocol received approval by the Ethics Committee at Witten/Herdecke University (No. 64/2022). The participants informed about the freedom to quit the study whenever they want in agreement with the Helsinki declaration of medical research and confirmed that the data may be used for scientific research. Clinicaltrials.gov registered the study with the ID number: NCT06438354 as a pilot RCT. All participants had been diagnosed with residual periodontal pockets either exceeding 5 mm or equalling 4-5 mm in PPD with bleeding on probing (BOP) at the first re-evaluation after step 2 periodontal therapy [19]. Before enrolment, all patients signed a written informed consent form. After signing the consent form, patients were randomly allocated to either test group (group A, subgingival instrumentation plus adjunctive protocol) or control group (group B, subgingival instrumentation only).

The study was conducted according to current standards of clinical research (CONSORT guidelines: http://www.consort-statement.org/) [20] between August 2022 and October 2023 in a private dental office in Munich, Germany.

Inclusion and exclusion criteria

The inclusion criteria encompassed systemically healthy adult individuals who were previously diagnosed with periodontitis according to the clinical practice guideline and who had completed step 1 and 2 therapy initially presenting with untreated periodontitis at stage 3 or 4 (Fig. 1) [8]. In particular, the study focused on persistent periodontal pockets characterized by a probing pocket depth (PPD) of ≥ 5 mm or 4 mm with positive BOP assessed at first re-evaluation following step 2 treatment. The exclusion criteria comprised individuals with unregulated T2DM with an HbA1c scores $> 7.5\%$, other chronic conditions such as rheumatoid arthritis, or pregnancy and lactating. The allocation of sites for therapy per patient was not constrained, and there were no restrictions on the location of residual sites. Both, single- and multi-rooted teeth were considered for the study.

Sample size calculation

At setting the protocol for the study, no numbers for the proposed therapy option were available from literature so far. Conceptualization of a pilot clinical investigation estimated a relevant average difference in CAL of 1 mm between both

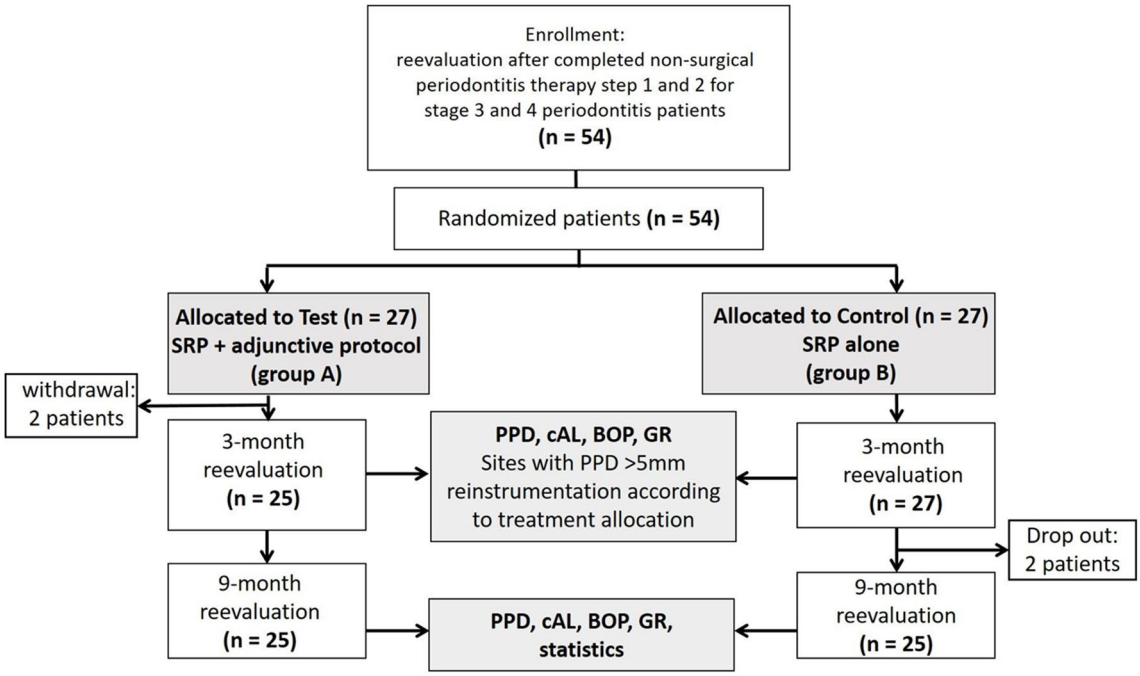

Fig. 1 The study outline

study groups with a standard deviation of 1 mm. Thus, the number of necessary cases to demonstrate the anticipated effect with a power of at least 80% was estimated with 24 per group. Accounting for possible dropouts during the study period, the number of patients was increased to 27 in each group.

Randomization and blinding

Fifty-two patients were randomized into two treatment groups. A computer-generated randomization table was created. Patients were assigned unique numbers from 1 to 52, and 2 sets of randomized numbers were generated (26 for control group subjects and 26 for test). Allocation concealment was performed using sealed envelopes to be opened before SPT treatment after the probing depth was re-evaluated. The generation of the random sequence allocation and the assignment of participants to interventions were performed by the investigator, who performed the treatment herself (L.B.). Thus, there was no blinding of the investigator possible in study setting.

Treatment protocol

One calibrated investigator (L.B.) performed the subgingival instrumentation under local anesthesia with 4% articaine (Ultracain DS, Sanofi, Germany). The calibration was achieved by measuring the PPD and Recession at the phantom model for diagnosing periodontitis (A-PB Frasaco, Germany) in three separate cycles. The study coordinator (A.F.) controlled the evaluated numbers, calibration fulfilled the requirements since overall agreement between three assessments exceeded 90% level.

Patients enrolled have been re-instructed in oral hygiene measures and treated by the operator (L.B.). The approximal plaque index (API) documented the progress in improving adequate oral hygiene performed by the patients at home [21]. The change in the scores of Approximal Plaque Index (API) compared the level assessed at evaluation after initial therapy with the scores assessed during the study.

The mechanical treatment regimen was identical for sites from both the test and control groups, respectively. In brief, sites underwent subgingival instrumentation by using Gracey curettes (HU-Friedy Group, Chicago, USA), ultrasonic instruments (SONICflex; KaVo, Biberach an der Riß, Germany), and glycine powder air polishing (Airflow; EMS, Vallée de Joux, Switzerland). In the test group (group A), each site intended to treat received subgingivally applied sodium hypochlorite cleaning gel (Perisolv; Regedent AG, Zürich, Switzerland) for 30 s before starting SI. Thereafter, a thorough mechanical debridement of the biofilm followed. The application of sodium hypochlorite gel repeated twice to enhance decontamination effect. An explorer probe (EXS3A6, HU-Friedy Group, Chicago, USA) verified the desired and appropriate instrumentation result. Concomitantly, the sites received 0.2–0.3 ml of xHyA (hyaDENT BG, Regedent AG, Zürich, Switzerland) up to the gingival margin.

Patients received instructions to continue oral hygiene without any restriction after completing the SPT visit regardless treatment group allocation. Subsequently, within the following 7 days, a second application of xHyA (0.2–0.3 ml) was placed subgingivally alongside with the control of oral hygiene. At three months re-evaluation, the treatment was repeated according to patient's allocation in sites presenting with persistently constant PPD ≥ 5 mm and positive BOP. The final re-evaluation took place 9 months post-treatment (Fig. 1).

Statistical analysis

Descriptive statistics were applied for the metrical variables pocket probing depth (PPD), clinical attachment level (CAL), and recession, including mean, standard deviation, median, minimum, and maximum. For nominal data (BOP), percentages were calculated to summarize the data. The primary parameter was CAL, while PPD, BOP and Rec applied as secondary parameters. To determine univariate differences between groups Analysis of Variance (ANOVA) or Chi-Square-Test in the case of nominal data were calculated. For the analysis of group effects repeated-measures ANOVA models were performed on the outcomes CAL, PPD, GR. In case of variance heterogeneity across time determined by Mauchly's test of sphericity, Greenhouse–Geisser adjusted testing was applied. Treatment group by time interaction effects were additionally quantified using 95% confidence intervals and partial eta square as effect size η^2. Values of 0.01–0.06, 0.06–0.14, and more than 0.14 were interpreted as small, medium, or large effects, respectively. Statistical analysis performed with the IBM SPSS 27 software package (IBM Corp.).

Outcomes displayed using estimated marginal means across the time points for both groups. For all analyses, a two-tailed significance level of $\alpha = 5\%$ was applied.

Results

The present study enrolled 52 patients with 1448 sites intended to treat after two patients allocated to group A withdrawn participation agreement. The mean age was 58.6 ± 12.4, with 20 patients being females (40%) and 30 males (60%). All patients were normoglycemic, and 15 patients (30%) were smokers. The oral hygiene improved continuously from the baseline level to the final examination 9 months after starting this trial. Figure 2 displays the positive development of oral hygiene performance at mean level for both groups. Both groups exhibited homogeneity in terms of age, gender, initial number of sites (31.38 ± 21.35 vs. 28.63 ± 17.60, respectively; $p = 0.622$), and BOP frequency at baseline evaluation (Table 1).

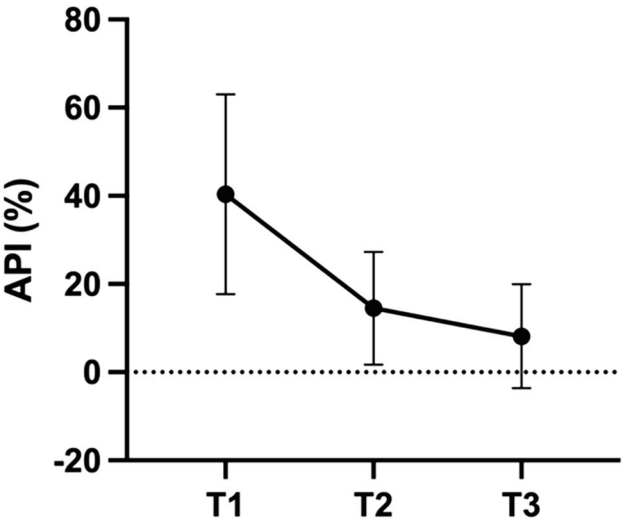

Fig. 2 Development of API throughout the observation period

According to the randomized allocation, 764 sites received the adjunctive protocol (treatment group A), while 684 sites received the subgingival treatment without adjunctives (treatment group B). Summarizing the treated sites by patient allocation, 25 patients underwent treatment by option A and 27 patients by option B. Fifty out of 52 patients completed the study; two dropouts occurred in group B following the 3-month reevaluation. One patient relocated, while the other one had to be excluded due to inadequate compliance. Healing was uneventful in all patients; participants did not report any unexpected adverse events, and the investigator observed neither.

At the outset (T1), baseline mean values for the primary parameter, CAL indicated no significant variance between the two groups (5.85 ± 1.42 mm for group A vs. 6.07 ± 1.59 mm for group B), ($p = 0.105$). Nevertheless, as the intervention effect unfolded, Group A consistently demonstrated a remarkable enhancement in CAL compared to Group B at both 3- and 9-month visits (T2: 4.44 ± 1.37 mm and T3: 3.76 ± 1.18 mm for group A vs. T2: 4.85 ± 1.66 mm and T3: 4.59 ± 1.70 mm for group B, respectively), with these distinctions holding statistical significance ($p = 0.001$).

Similarly, the means for PPD showed no statistically significant divergence between Group A and Group B (T1: 4.74 ± 0.99 mm group A vs. 4.69 ± 1.01 mm group B) ($p = 0.193$, Table 2). As the study progressed, both groups exhibited a notable and significant reduction in PPD (T2: 3.50 ± 1.03 mm in group A and 3.35 ± 1.08 mm in group B; T3: 2.94 ± 0.82 mm and 3.14 ± 1.01 mm, respectively) (Table 2). However, Group A exhibited a more substantial reduction than Group B at both, the 3- and 9-month evaluations and these disparities were statistically significant at the 9-month evaluation ($p = 0.001$, Table 4).

Table 1 Demographic data from two groups of patients with controlled homogeneity

	Group A ($n=25$)	Group B ($n=25$)	Total ($N=50$)	p-value
Age				
Mean ± SD	60.6 ± 11.3	56.4 ± 13.5	58.6 ± 12.4	0.232
Median	58	55.5	56.5	
Min	41	29	29	
Max	82	82	82	
Gender				
Male	19 (73.1%)	11 (45.8%)	30 (60.0%)	0.082
Female	7 (26.9%)	13 (54.2%)	20 (40.0%)	
Smoker				
Yes	7 (26.9%)	8 (33.3%)	15 (30.0%)	0.760
No	19 (73.1%)	16 (66.7%)	35 (70.0%)	
Number of measuring points				
Mean ± SD	31.38 ± 21.35	28.63 ± 17.60	30.06 ± 19.49	0.622
Median	27.5	25.5	26.0	
Min	5	8	5	
Max	81	82	82	

Table 2 Alterations in clinical parameters between BL (T1), 3- (T2) and 9-month (T3) visit

	Group A			Group B			p-value; partial η^2
	T1	T2	T3	T1	T2	T3	
GR							
Mean ± SD	1.12 ± 0.95	0.95 ± 0.88	0.81 ± 0.82	1.38 ± 1.14	1.51 ± 1.15	1.48 ± 1.15	< 0.001; 0.059
Median	1.00	1.00	1.00	1.00	1.00	1.00	
Minimum	0	0	0	0	0	0	
Maximum	5	5	5	5	5	5	
PPD							
Mean ± SD	4.74 ± 0.99	3.50 ± 1.03	2.94 ± 0.82	4.69 ± 1.01	3.35 ± 1.08	3.14 ± 1.01	< 0.001; 0.014
Median	4.00	3.00	3.00	4.00	3.00	3.00	
Minimum	2	1	1	4	1	1	
Maximum	12	6	5	10	9	8	
CAL							
Mean ± SD	5.85 ± 1.42	4.44 ± 1.37	3.76 ± 1.18	6.07 ± 1.59	4.85 ± 1.66	4.59 ± 1.70	< 0.001; 0.031
Median	6.00	4.00	4.00	6.00	5.00	4.00	
Minimum	4	1	1	4	2	1	
Maximum	13	9	9	14	12	11	
BOP							
Mean ± SD	0.78 ± 0.42	0.17 ± 0.48	0.11 ± 0.32	0.78 ± 0.42	0.21 ± 0.41	0.13 ± 0.34	0.360; 0.001
Median	1.00	0.00	0.00	1.00	0.00	0.00	
Minimum	0	0	0	0	0	0	
Maximum	1	9	1	1	1	1	

Gingival Recession (GR) scores were significantly different between Group A and B at baseline. Throughout the 9-month evaluation, Group A displayed insignificant but rather positive changes, while Group B showed little progression in GR depth from Baseline to 3 months, remaining constantly at the same level for a further 6 months. The intergroup difference in mean GR level at both evaluations was statistically significant ($p=0.001$) (Fig. 3a-d).

Conversely, the BOP frequency yielded no statistically significant difference between the two groups at any time point, neither at baseline, at 3-months, nor 9-month evaluation, with p-values of 0.796, 0.175, and 0.339, respectively. Details of the clinical parameters and their alterations presented in Table 2. The frequency of pocket closure (PPD ≤ 4 mm / BOP negative) after the 9-month observation period was indifferent in the shallow pockets (4-5 mm). Interestingly, the pocket closure rate decreased in the deeper pockets but was still higher in the treatment group. In the very deep pockets > 7 mm, group A exhibited

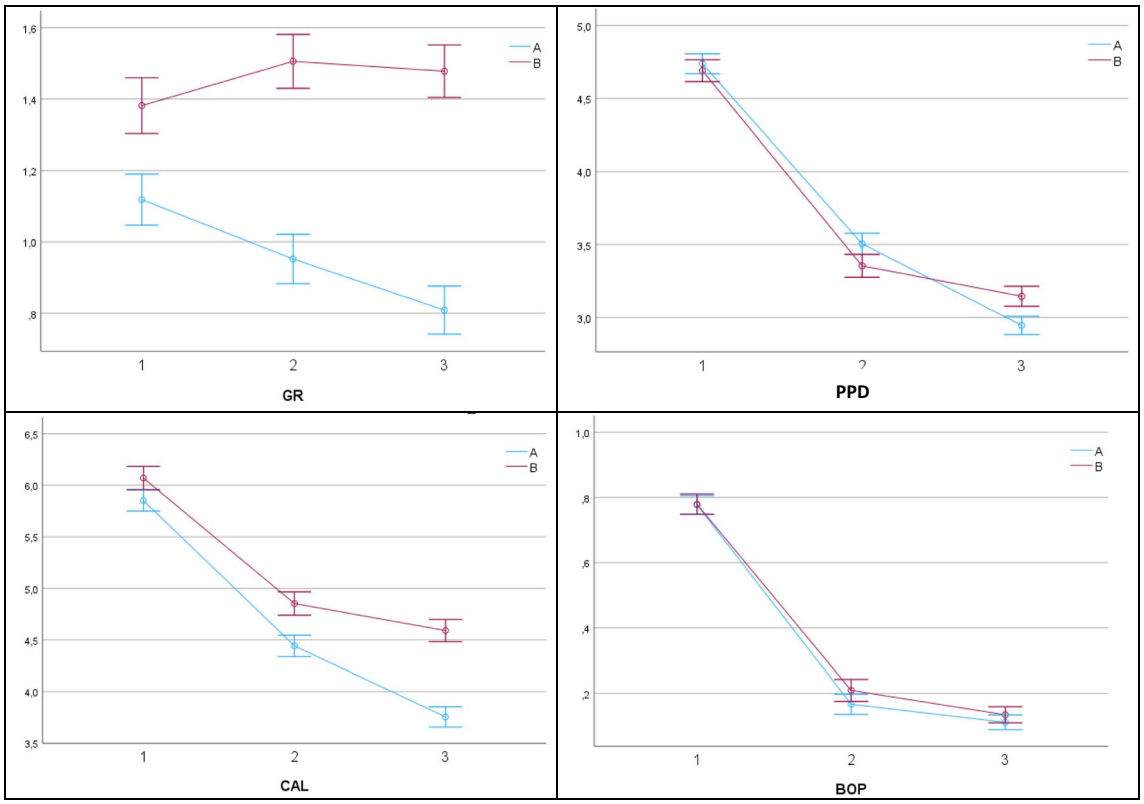

Fig. 3 **a-d** Clinical parameters and their alterations during 9-month observation, estimated Marginal Means and their 95% confidence intervals as computed by the General Linear Model

Table 3 Pocket closure effect per treatment group based on residual PPD ≤ 4 mm and negative BOP at 9-month reevaluation stratified by initial PPD at patient enrolment

BL PPD (mm)	N sites treated per subgroup	N sites treated per allocation to A or B	N sites applying to pocket closure	Rate of pocket closure effect
Severe ≥ 8	**36**	**17** sites group A	**16**	94%
		19 sites group B	**8**	42%
Moderate = 6–7	**214**	**126** sites group A	**93**	73%
		88 sites group B	**58**	66%
Shallow = 4–5	**1248**	**671** sites group A	**633**	94%
		577 sites group B	**521**	90%

a pocket closure rate of 94%, while the control group only exhibited 42% pocket closure (Table 3).

The inter-group comparison of the means for all four parameters at any time point of the study displayed by Table 4, showing the respective level of significance and the partial effect size (**partial η^2**).

Discussion

The present study evaluated the clinical effect of the adjunctive combination of sodium hypochlorite/amino acid gel and xHyA for treating residual inflamed periodontal

Table 4 Between group effects for the different time points

	Group A vs. Group B		
	T1	T2	T3
GR			
p-Value	< 0.001	< 0.001	< 0.001
partial η^2	0.009	0.057	0.090
PPD			
p-Value	0.193	< 0.001	< 0.001
partial η^2	0.001	0.010	0.009
CAL			
p-Value	0.105	< 0.001	< 0.001
partial η^2	0.002	0.009	0.066
BOP			
p-Value	0.796	0.175	0.339
partial η^2	< 0.001	0.001	0.001

pockets during SPT in a private praxis environment. Clinically, the treatment and the healing were uneventful in all cases. The study results reveal that repeated instrumentation during SPT was an effective option to further improve clinical periodontal parameters in both groups. However, the adjunctive protocol investigated in this study yielded better results than the control group regarding attachment level gain. Intriguingly, the pocket closure rates were significantly higher in test group as compared to the controls when looking at deep residual pockets (Table 3), indicating that the adjunctive protocol was highly beneficial for deep residual sites. Thus, the response in sites designated for surgical intervention due to scores assessed at re-evaluation emphasized the probability of waiving surgery by means of meticulous instrumentation supported by adjunctive use of hypochlorite/amino acids and xHyA.

The three-months evaluation of periodontal conditions after completion of initial periodontal treatment applies to German regulations for treating periodontitis within the health insurance system, but is also backed up by the recent meta-analysis [22]. The data extracted from 29 RCT studies leave no room for the assumption the re-evaluation performed three months after completion of initial subgingival instrumentation may impair ongoing healing. While our group set up the protocol and conducted the clinical part for the step 3 SPT treatment in a private praxis environment, the group from Lithuania used the same adjunctive protocol at step 2 treatment of periodontal pockets [23]. The authors reported significantly better PD reduction over an observation period of 6 months for the test group compared to controls (2.9 ± 0.4 vs 1.8 ± 0.6 mm, $p < 0.001$, respectively). Similarly, mean CAL gain was statistically higher in the test group compared to the control one (test: 2.6 ± 0.5 vs. control: 1.6 ± 0.6 mm, $p < 0.001$). The notable pocket closure rate in sites with PPD exceeding 6 mm dropped from total of 298 (8.7%) to 4 (0.1%) in test group ($p = 0.003$), and from 277 (7.6%) to 35 (1.0%) in the controls. The declining detection rates for five targeted periodontal pathogens in samples obtained from subgingival compartment at three clinical visits accordingly to clinical examinations reflected the abovementioned benefit of the adjunctive protocol applied at step 2 periodontal therapy. The detection frequencies for *A.actinomycetemcomitans*, *P. gingivalis*, *Pr. Intermedia*, *T. denticola* and *T. forsythsia* were significantly smaller in the test group vs. controls in 6-month samples [24]. Thus, the counts of the targeted putative periodontal pathogens showed significantly and continuously reduced scores in sites that showed the greatest clinical response in the test group, whereas the controls demonstrated a kind of rebound in colonization by the same microorganisms during the observation period of 6 months. The conformity of clinical and microbiological outcomes underscored the benefit of the combined hypochlorite/ amino acid gel use followed by xHyA application after completed NSPT. Although this study addressed another step in the treatment flow of periodontal pockets, our clinical outcome agreed with the data cited above pointing out the universal benefit proposed approach unfolds in a non-surgical treatment for deep periodontal pockets. The clinical parameters, PPD, CAL, BOP improved significantly from baseline to 6-months reevaluation in a case series which used same adjunctives during step 2 therapy [23].

Similar clinical results we recorded in this study over a period of 9 months post-op. However, the treatment population was recruited from the patients who presented with residual active periodontal pockets and were referred to either step 3 or 4 – the SPT. Intriguingly, the pocket closure rates were substantially higher in the treatment group, while patients with shallow pockets did not seem to benefit from the adjunctive treatment. This observation may partly explain the contradicting results for xHya-supported surgical and non-surgical treatment protocols [25]. A very similar rate in BOP frequency in both groups consistently recorded from baseline to latest re-evaluation may be related to the masking effect which smoking induces on bleeding on probing frequency by duration of the habit [26]. In our study, about 30% of participants in both groups were smokers.

In vitro studies, sodium hypochlorite has proven effective against gram-negative species-dominated biofilms [27]. In addition, coating dentin surfaces with hypochlorite/amino acid also promoted the proliferation of periodontal ligament fibroblasts on these surfaces in vitro [28]. Moreover, subgingivally applied as a singular adjunctive for treating periodontitis, the hypochlorite/amino acid gel has been shown to enhance antimicrobial and therapeutic effects expressed by improved clinical parameters in randomized clinical studies [29, 30]. However, in a randomized clinical trial, the additional use of sodium hypochlorite

gel did not affect the outcomes of manual or ultrasonic subgingival instrumentation in a limited group of patients at the SPT stage [31].

Hyaluronic acid, a ubiquitous component in mammalian tissues, exhibits a versatile spectrum of properties. Studies have demonstrated that HA has a bacteriostatic effect, contributing to minimizing bacterial contamination in surgical wounds [32]. Additionally, HA has a fungistatic effect on the growth of Candida albicans [33]. However, using xHyA as an adjunct to mechanical treatment of periodontal pockets failed to unleash a greater effect than subgingival instrumentation alone in a 12-month randomized non-surgical study [14].

Results from animal studies with a surgical approach proved the potential of xHyA to support new attachment formation in a recession canine model, an acute intrabony defect, and an acute furcation Grade 3 model. The microscopic outcome in all these experiments proved new cementum, ligamentum, and bone formation, respectively. Histomorphometric assessment revealed a clinically relevant amount of newly organized tissues in sites treated with xHyA [34–36]. HA presence can also promote osteosynthesis by enhancing mesenchymal cell differentiation in critical-sized bone defects and has been shown to stimulate osteoblasts, underscoring a great potential for periodontal regeneration [37, 38].

Accordingly, a randomized controlled trial comparing the efficacy of enamel matrix derivatives and xHya in treating contained three-wall intrabony defects reported equal regenerative outcomes for both biologics [39]. Effective decontamination of the root surface and periodontal pocket is a prerequisite for effective regeneration and represents a major rationale for flap elevation. Same requirement is a particular challenge for achieving regenerative healing by non-surgical approach. The recent animal study performed in chronic infected intrabony pockets by means of non-surgical subgingival instrumentation corroborated at histomorphometrical level the regenerative pattern of tissue formation if the adjunctive hypochlorite/amino acid and xHyA treatment was applied [40].

Naturally, this study exhibits some limitations. Because it is a single-center investigation in a private clinic involving just one investigator, a chance of investigator bias is inherent to this study. Moreover, radiographic validation of the clinical findings is warranted in future studies.

Nonetheless, the study's findings were clinically relevant, advocating for incorporating proposed adjunctive therapy in residual periodontal pocket management. Moreover, the high pocket closure rate of deep sites indicates that the adjunctive use of sodium hypochlorite and xHya may circumvent surgical intervention. Future studies must, therefore employ more independent and blinded investigators to increase the generalizability of findings.

Conclusions

Within the limitations of this mono centre RCT study we consider the proposed adjunctive treatment protocol sufficient in improving clinical conditions in persisting residual periodontal pockets at relevant level during SPT visit. Thus, patients showing off with residual deep sites at reevaluation may carry a sustained benefit from such extended therapy effort.

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Author contribution L.B. conducted clinical research, data management, writing the manuscript, A.F. conception of the study protocol, supervision of the study, data collection, writing the manuscript, T.O. data management, statistics, data interpretation, proof reading the manuscript, D.D. writing the manuscript, data interpretation, graphical work.

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Data availability The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request. Data are located in controlled access data storage at Witten/Herdecke University, Department of Periodontology.

Declarations

Competing interest The authors declare no competing interests.

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RESEARCH

In vitro activity of hyaluronic acid and human serum on periodontal biofilm and periodontal ligament fibroblasts

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Abstract

Objectives A beneficial effect of cross-linked hyaluronic acid (cHA) on periodontal wound healing and regeneration has recently been demonstrated. The present in vitro study was designed to obtain deeper knowledge on the effect of cHA when applied in the gingival sulcus (serum-rich environment) during non-surgical periodontal therapy.

Materials and methods The influence of cHA, human serum (HS), and cHA/HS on (i) a 12-species biofilm formation, (ii) the adhesion of periodontal ligament fibroblasts (PDLF) to dentine surface, (iii) the expression and secretion of interleukin-8, and (iv) the expression of receptors of HA in PDLF and gingival fibroblasts (GF) were evaluated.

Results At 4 h of biofilm formation, cHA and HS in combination (cHA/HS) slightly decreased the colony-forming unit counts in biofilm whereas the metabolic activity of biofilm was reduced in all test groups (cHA, HS, cHA/HS) vs. control. At 24 h, the quantity of biofilm was reduced in all test groups vs. untreated control. The test substances did not affect adhesion of PDLF to dentin. HS increased the expression of IL-8 by PDLF and GF which was partially downregulated by cHA. HS and/or cHA promoted the expression of the HA receptor RHAMM in GF but not in PDLF.

Conclusions In summary, the present data indicate that serum neither negatively affect the activity of cHA against periodontal biofilm nor had any unwanted influence on the activity of PDLF.

Clinical relevance These findings lend additional support for the positive effects of cHA on cells involved in periodontal wound healing, thus pointing to its potential use in non-surgical periodontal therapy.

Keywords Periodontal therapy · Cross-linked hyaluronic acid · Periodontal ligament fibroblasts · Gingival fibroblasts · Interleukin-8 · Antibiofilm activity

Introduction

Hyaluronic acid (HA), also named hyaluronan, is a glycosaminoglycan and a major component of the extracellular matrix of vertebrate tissues, abundant in almost all body fluids such as synovial fluid or serum [1]. It is synthesized as a high-molecular weight polymer of 1000–6000 kDa, but can be degraded to low-molecular weight of less or equal 250 kDa or further fragmented to oligos [1]. In medicine, HA has become increasingly important as formulations used in wound healing, the treatment of osteoarthritis or of respiratory and urinary tract infections, and in tissue and regenerative medicine [2]. With respect to the oral cavity, HA is present in saliva [3], gingival crevicular fluid [4], and the soft periodontal tissues [5].

Periodontitis, a disease leading to the destruction of the tooth-supporting tissues, is characterized by an interaction of a dysbiotic biofilm with host response leading to an ongoing inflammatory state [6–8]. Therapy of periodontitis always includes the removal of the subgingival biofilm [9]. The subgingival area of a teeth is bathed in the gingival crevicular fluid, which corresponds to a serum transudate in periodontal health and a serum exudate in periodontal disease [10]. It contains many factors, e.g., immunoglobulins, antimicrobial peptides, or proteases, being involved in the immune response [11]. Periodontal ligament fibroblasts play a special part in periodontal tissue regeneration in the first place by establishing a new attachment [12]. Moreover, they are central players in innate immunity as in inflammation

Sigrun Eick and Alexandra Stähli both share the last position.

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they produce many mediators including proinflammatory cytokines such as interleukin (IL)-8 [13].

In vitro data have shown that a cross-linked hyaluronic acid (cHA) enhanced the expression of genes encoding type III collagen and transforming growth factor-β3, characteristic of scarless wound healing. Moreover, the cHA upregulated the expression of genes encoding pro-proliferative, pro-migratory, and proinflammatory factors and positively influenced the proliferative, migratory, and wound healing properties of different cell types involved in periodontal wound healing/regeneration [14]. These positive biologic effects of cHA on periodontal ligament cells have recently been confirmed in a series of experimental studies providing histological evidence for periodontal regeneration in intrabony, recession, and furcation defects following regenerative surgery and application of cHA [15–17]. Results from controlled clinical studies have provided further evidence on the potential clinical relevance of using cHA in regenerative periodontal surgery in intrabony and recession defects [18, 19].

Systematic reviews underlined a beneficial effect of HA on clinical outcomes (periodontal probing depth (PPD) reduction, less bleeding on probing (BOP), clinical attachment level (CAL) gain) of surgical and non-surgical periodontal therapy [20, 21]. In the included studies, different formulations of high-molecular weight HA and of different origins were applied [21]. In a recent RCT, a gel formulation was used which contained mainly cross-linked high-molecular weight HA added by a small amount of natural high-molecular weight HA [22]. After 3 months of non-surgical periodontal therapy, differences in BOP and PPD reduction were clearly in favor of the HA-treated group [22]. Using adjunctively the gel formulation in residual pockets resulted in by trend (not statistically significant) better results vs. instrumentation alone after 12 months [23].

The aim of this in vitro study was to get deeper knowledge of the effect of cHA when applied in the gingival sulcus during non-surgical periodontal therapy. The focus was on the interaction of microorganisms and periodontal fibroblasts against the background that serum is an essential component of gingival crevicular fluid. We analyzed the influence of cHA on (i) biofilm formation, (ii) the adhesion of periodontal ligament fibroblasts to tooth surface, (iii) the expression and secretion of interleukin-8, and (iv) the expression of receptors of HA in periodontal fibroblasts.

Materials and methods

HA and human serum preparation

As hyaluronic acid formulation a commercially available product (Hyadent BG®, Regedent AG, Zurich, Switzerland) was used. According to the manufacturer's information, the product (cHA) contains 16 mg cross-linked HA (molecular weight of about 1000 kDa) and 2 mg natural HA per ml.

Human serum (HS) was purchased from Sigma-Aldrich (Merk KGaA, Darmstadt, Germany). In the assays, cHA was used in concentrations of 12.5 mg/ml (0.225 mg/ml HA), 25 mg/ml (0.45 mg/ml HA), and 50 mg/ml (0.9 mg/ml HA) and HS in concentrations of 12.5 mg/ml, 25 mg/ml, and 50 mg/ml. When cHA was used in combination with serum (cHA/HS), the respective concentrations each of both were 12.5 mg/ml, 25 mg/ml, and 50 mg/ml. A 0.9% w/v NaCl solution was the negative control.

Microorganisms

A 12-species periodontal biofilm was used in this study:

1. *Streptococcus gordonii* ATCC 10558
2. *Actinomyces naeslundii* ATCC 12104
3. *Fusobacterium nucleatum* ATCC 25586
4. *Campylobacter rectus* ATCC 33238
5. *Parvimonas micra* ATCC 33270
6. *Eikenella corrodens* ATCC 23834
7. *Treponema denticola* ATCC 35405
8. *Prevotella intermedia* ATCC 25611
9. *Capnocytophaga gingivalis* ATCC 33624
10. *Porphyromonas gingivalis* ATCC 33277
11. *Tannerella forsythia* ATCC 43037
12. *Filifactor alocis* ATCC 33099

All strains (except for *T. denticola* which was maintained in Mycoplasma broth (BD, Franklin Lake, NJ)) were cultured on Schaedler agar plates (Oxoid, Basingstoke, UK) with 5% sheep blood, in an anaerobic incubator or with 5% CO_2 (*S. gordonii*) at 37 °C. The bacteria were suspended in 0.9% w/v NaCl according to McFarland 4. One part *S. gordonii* was mixed with two parts *A. naeslundii,* and four parts of the other nine species.

Cell culture

Human gingival fibroblasts (GF) and human periodontal ligament fibroblasts (PDLF) were harvested from freshly extracted and donated teeth from patients who had been informed of the use of their teeth for research purposes and signed written agreement. As these biomaterials were irreversibly anonymized, no additional approval of the Cantonal ethical committee (KEK) was needed according to the respective guidelines.

The procedure was as described recently [24, 25]. GF and PDLF were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS;

Invitrogen). For experiments, cells were used between the third and fifth passage. Cells from two donors were included.

All cells were incubated with 5% CO_2 at 37 °C.

Activity on periodontal biofilm formation

First, wells of 96-well plates were coated with each 10 µl 1.5% bovine serum albumin (BSA, SERVA Electrophoresis GmbH, Heidelberg, Germany) in phosphate-buffered saline (PBS) for 1 h to generate a proteinaceous layer. Then, 10 µl of test substances (cHA, HS, cHA/HS, final concentration each 50 mg/ml) and the control were added for 30 min incubation. Thereafter, microbial suspension mixed with cultivation broth (Wilkins–Chalgren broth, Oxoid, Basingstoke, UK) in a volume ratio of 1:9 was additionally added, i.e., 200 µl per well. Thereafter, the plates were incubated in an anaerobic incubator, 37 °C for 4 h or 24 h.

At 4 h and 24 h, three different aspects of periodontal biofilm formation were measured: (a) colony-forming units (cfus), (b) biofilm mass, and (c) metabolic activity. Then, following a short, careful washing, 100 µl of 0.9% w/v NaCl were added. Biofilms were scraped from the surface and mixed. One aliquot of the suspension was serially diluted, plated on Schaedler agar plates. The cfus were counted after 8 days of anaerobic incubation. Biofilm quantity was measured by using crystal violet staining, and metabolic activity was determined by Alamar blue staining assay as described before [25].

Activity on adhesion of PDL fibroblasts to dentine specimens

The dentin discs (about $4 \times 4 \times 1$ mm) were prepared as described recently [26]. The teeth were donated from patients who had been informed of the use of their teeth for research purposes and signed written agreement. As these biomaterials were irreversibly anonymized, no additional approval of the Cantonal ethical committee (KEK) was needed according to the respective guidelines. The dentine discs were placed in 24-well plates in the laminar flow. Then the discs of the serum groups (HS, CHA/HS) were coated with 10 µl of serum (undiluted) for 5 min in the laminar flow and thereafter those of the cHA groups (cHA, CHA/HS) with 10 µl of cHA (undiluted for 5 min). The controls were left uncoated.

Detached PDL fibroblasts were suspended in cell culture (with 1% FBS) to a density of 5×10^6/ml. After a short dipping of the test specimens into 0.9% w/v NaCl, each 1 ml of the cell suspension were added per well. The plates have been incubated with 5% CO_2 for 72 h. Then after short washing and fixing the cells with methanol, the attached cells were counted. The results represent the mean of 10 fields (mm^2). The statistical unit was the dentine specimen.

Release and expression of interleukin-8 and HA receptors by gingival and PDL fibroblasts

For determining cytokine level, each well of 48-well plates was covered with 25 µl of the test substances (final concentrations 12.5 mg/ml, 25 mg/ml, and 50 mg/ml each) for 30 min incubation (RT). Afterwards, 225 µl cell suspension was added at a density of 5×10^5 cells/well (GF and PDLF). After 18 h of incubation (37 °C, 5% CO_2), the media were collected and centrifuged. From the supernatants, the protein level of IL-8 was quantified by ELISA kits (R&D Systems Europe Ltd., Abingdon, UK) following the manufacturer's instructions.

For measuring mRNA expression of the cytokine IL-8 and also of the HA receptors (CD44, RHAMM, TLR2, and TLR4), GF and PDLF were seeded into 6-well plates at a density of 5×10^5 cells/well for 18 h. Then, after careful washing, culture medium with 0.5% FBS and the test substances in concentrations of each 25 mg/ml were added for 1 h. After 3 times PBS wash, total RNA was extracted following the instruction of innuPREP RNA Mini Kit 2.0 (Analytic Jena GmbH, Jena, Germany). Then, the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) was used to reverse 1000 ng RNA into cDNA. Quantitative RT-PCR was carried out by GoTaq® qPCR Master Mix (Promega) with the QuantStudio 3 Real-Time PCR System (Thermo Fischer, Waltham, MA, USA) to determine the mRNA expression level of the cytokine IL-8, and HA receptor genes (CD44, RHAMM). The primer sets are given in Table 1. Gene expression was normalized by GAPDH and analyzed by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All experiments were performed in at least two independent experiments in each quadruplicate (eight independent biological samples). Log_{10} transformation was used in the case of cfu counts.

Statistical analysis was performed with Kruskal–Wallis test and followed by Mann–Whitney *U* test (with Bonferroni correction) using SPSS 26.0 (IBM Corporation, New York, NY, USA). For qRT-PCR results, one-way ANOVA and Dunnett's multiple comparisons test were carried out by Graphpad Prism 9 (Graphpad Software, California, USA). Statistical significance was set at $p < 0.05$.

Table 1 Primer sequences used for qRT-PCR

Gene	Forward/reverse primers	Primer sequences 5'-3'	References
IL-8	F	GAG AGT GAT TGA GAG GTG GAC CAC	[27]
	R	CAC AAC CCT CTG CAC CCA GTT T	
CD44	F	GAC CTC TGC AAG GCT TTC AAT A	# M59040.1
	R	CAA AGG CAT TGG GCA GGT CT	
RHAMM	F	AGG ACC AGT ATC CTT TCA GAA ATC	# BC017793.1
	R	AGT GCA GCA TTT AGC CTT GC	
GAPDH	F	GAC AGT CAG CCG CAT CTT CT	[28]
	R	TTA AAA GCA GCC CTG GTG AC	

Results

Periodontal biofilm formation

In median, the untreated biofilm consisted of 6.59 log10 cfu at 4 h and of 8.90 log10 cfu at 24 h.

When applying test substances, there were only minor differences in the cfu counts. The highest difference vs. control was − 0.36 log10, when 50 mg/ml cHA/HS were applied at 4 h ($p < 0.001$; Fig. 1a). At 24 h, all test substances increased the cfu counts; however, differences were in median 0.10 log10 (cHA, $p = 0.483$) to 0.13 log10 (HS, $p = 0.015$).

In terms of biofilm quantity (Fig. 1b), at 4 h, both cHA and cHA/HS groups had higher values compared to the control group (each $p < 0.001$). The quantity of biofilm in HS group decreased ($p = 0.009$). However, at 24 h, the quantity of biofilm was reduced in all test groups vs. untreated control ($p < 0.001$ each).

In all test groups, the metabolic activity of the biofilm was reduced compared to the control at 4 h ($p < 0.001$ each). At 24 h, no difference was found anymore (Fig. 1c).

HA and attachment of PDL fibroblasts to dentin

An important step in the resolution of periodontal tissue destruction is a promoted adhesion of fibroblasts to tooth surfaces in the periodontal pocket. Here, the influence of HS and cHA on the number of adhered PDL fibroblasts was studied. There was a minor trend (not statistically significant) to a reduced attachment when the surface was coated with cHA. In case of coating with HS and cHA/HS, the numbers remained unchanged (Fig. 2).

HA receptors expression in oral fibroblasts

Two crucial HA receptor genes were checked in gingival and PDL fibroblasts. Each 25 mg/ml of test substances (HS, cHA) were used for the mRNA expression experiments.

The analyzed receptors (CD44, RHAMM) were expressed by both fibroblast types. An influence by the test substances was minor despite reaching in part statistical significance. In gingival fibroblasts (Fig. 3a), all test groups (cHA, HS, cHA/HS) increased the mRNA expression of RHAMM ($p = 0.003$, $p = 0.003$, $p = 0.001$). In PDL fibroblasts (3b), the receptors' mRNA expression did not statistically significantly differ among the groups.

Interleukin-8 expression in oral fibroblasts

Both mRNA expression and protein expression of interleukin-8 (IL-8) were measured in GF and PDLF, in which 25 mg/ml cHA and HS were used for mRNA expression whilst 5, 25, and 50 mg/ml were used for protein expression.

As shown in Fig. 4a and c, cHA decreased the mRNA expression of IL-8 in the two fibroblast types (GF: $p < 0.001$, PDLF: $p = 0.005$). At protein level, there was no statistically significant difference for any of the tested cHA concentrations vs. control neither with GF nor PDLF. In contrast, HS significantly increased IL-8 expression in GF and PDLF at the mRNA level ($p = 0.001$, $p = 0.009$). At protein level, results were accordingly, after each tested concentration of cHA higher IL-8 levels were measured vs. non-stimulated GF and PDLF cells (each $p < 0.001$). Also, when cHA was combined with serum, the released levels of IL-8 from GF and PDLF were always higher than from the control (each $p < 0.001$). When comparing the high levels of IL-8 after HS stimulation with those after cHA/HS, the combination with cHA decreased the mRNA expression (GF: $p < 0.001$, PDLF: $p = 0.002$) and also the protein expression (GF all concentrations $p < 0.001$, PDLF 12.5 mg/ml HS vs. 12.5 mg cHA/HS $p < 0.001$, 50 mg/ml HS vs. 50 mg cHA/HS $p = 0.001$).

Discussion

The present in vitro study has analyzed the effects of a commercial HA product on a periodontal biofilm and periodontal fibroblasts. A product topically used in non-surgical periodontal therapy should inhibit biofilm formation, and,

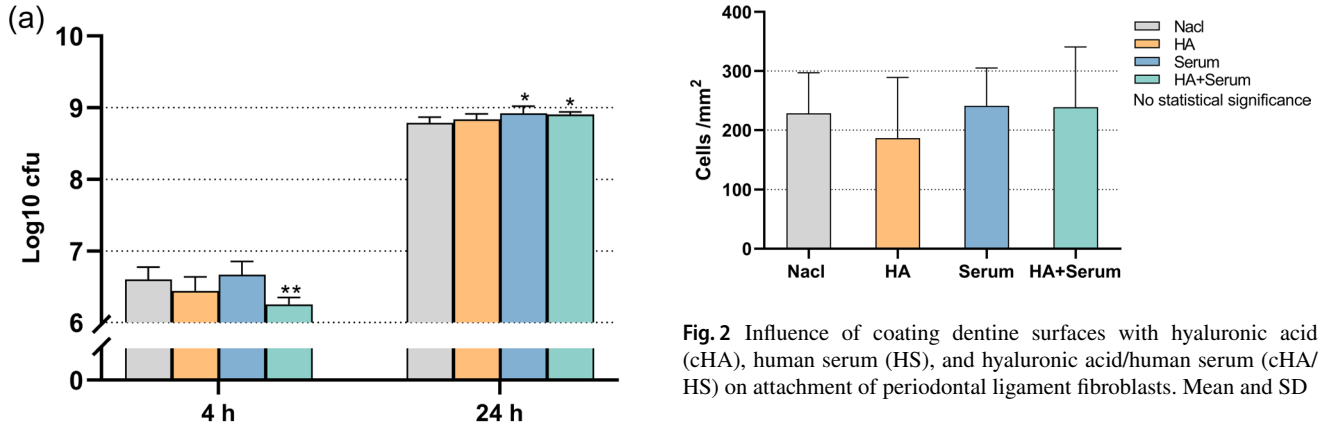

Fig. 2 Influence of coating dentine surfaces with hyaluronic acid (cHA), human serum (HS), and hyaluronic acid/human serum (cHA/HS) on attachment of periodontal ligament fibroblasts. Mean and SD

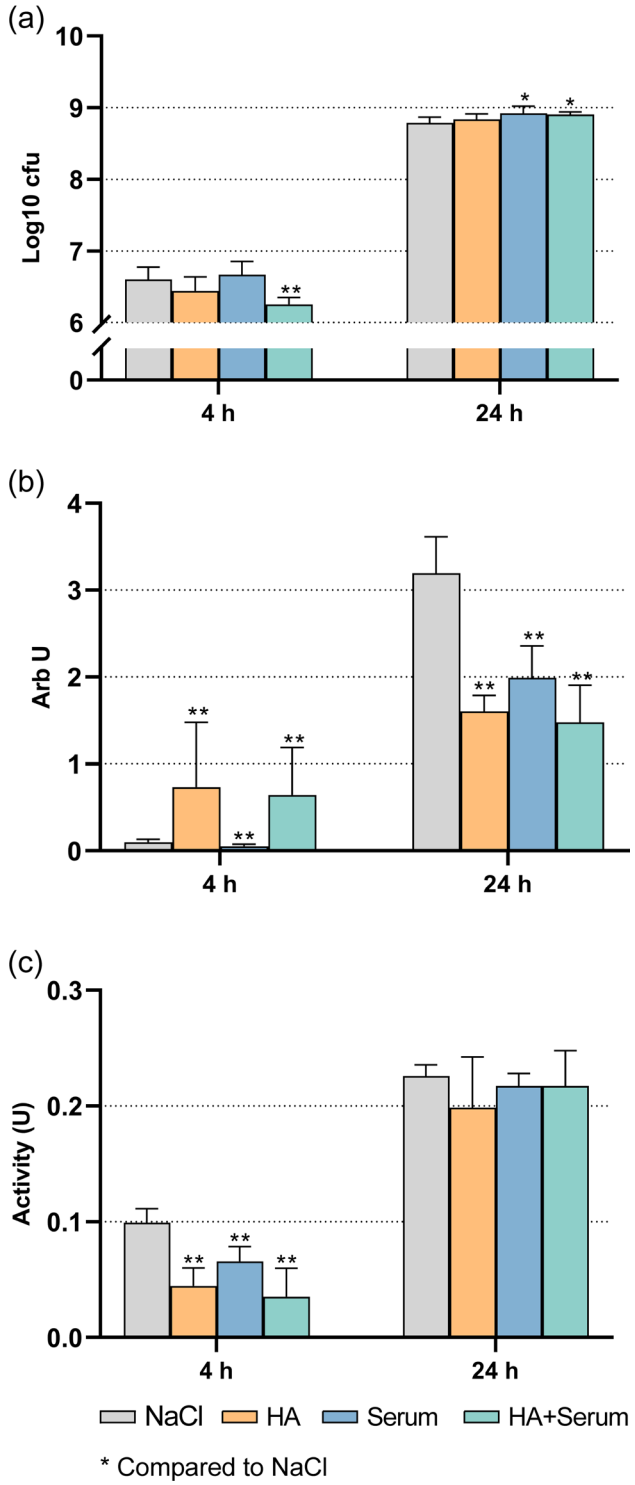

Fig. 1 Influence of coating with 50 mg/ml hyaluronic acid (cHA), human serum (HS), and each 50 mg/ml hyaluronic acid/human serum (cHA/HS) on periodontal biofilm formation after 4 h and 24 h. **a** Colony-forming units (cfu); **b** quantity; **c** metabolic activity. Median incl. 25 and 75 percentiles; *$p < 0.05$, **$p < 0.01$ vs. control

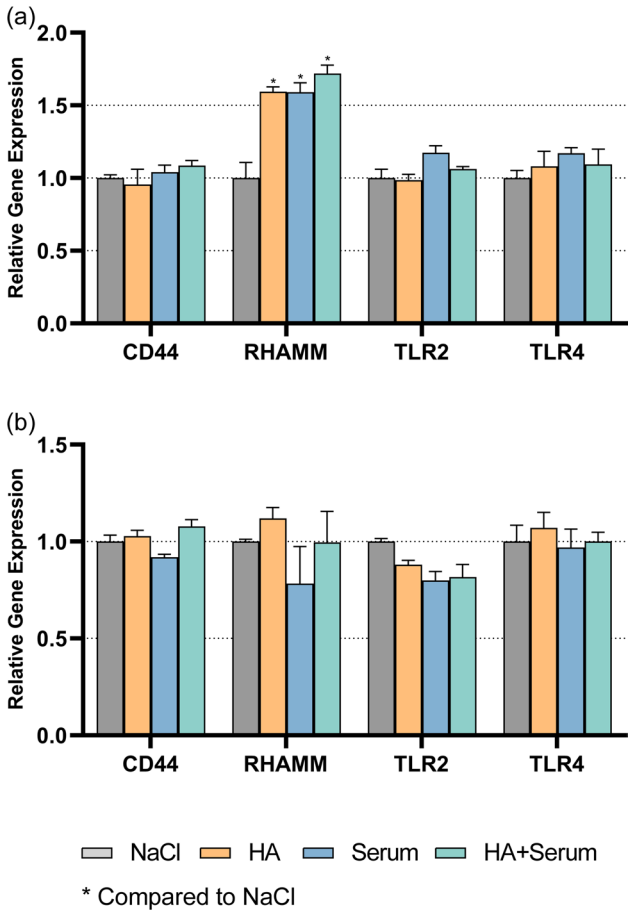

Fig. 3 Influence of 25 mg/ml hyaluronic acid (cHA), human serum (HS), and each 25 mg/ml hyaluronic acid/human serum (cHA/HS) on mRNA expression of hyaluronic acid receptors (CD44, RHAMM) in **a** gingival fibroblasts (GF), and **b** periodontal ligament fibroblasts (PDLF). Mean ± SD, *$p < 0.05$, **$p < 0.01$ vs. control

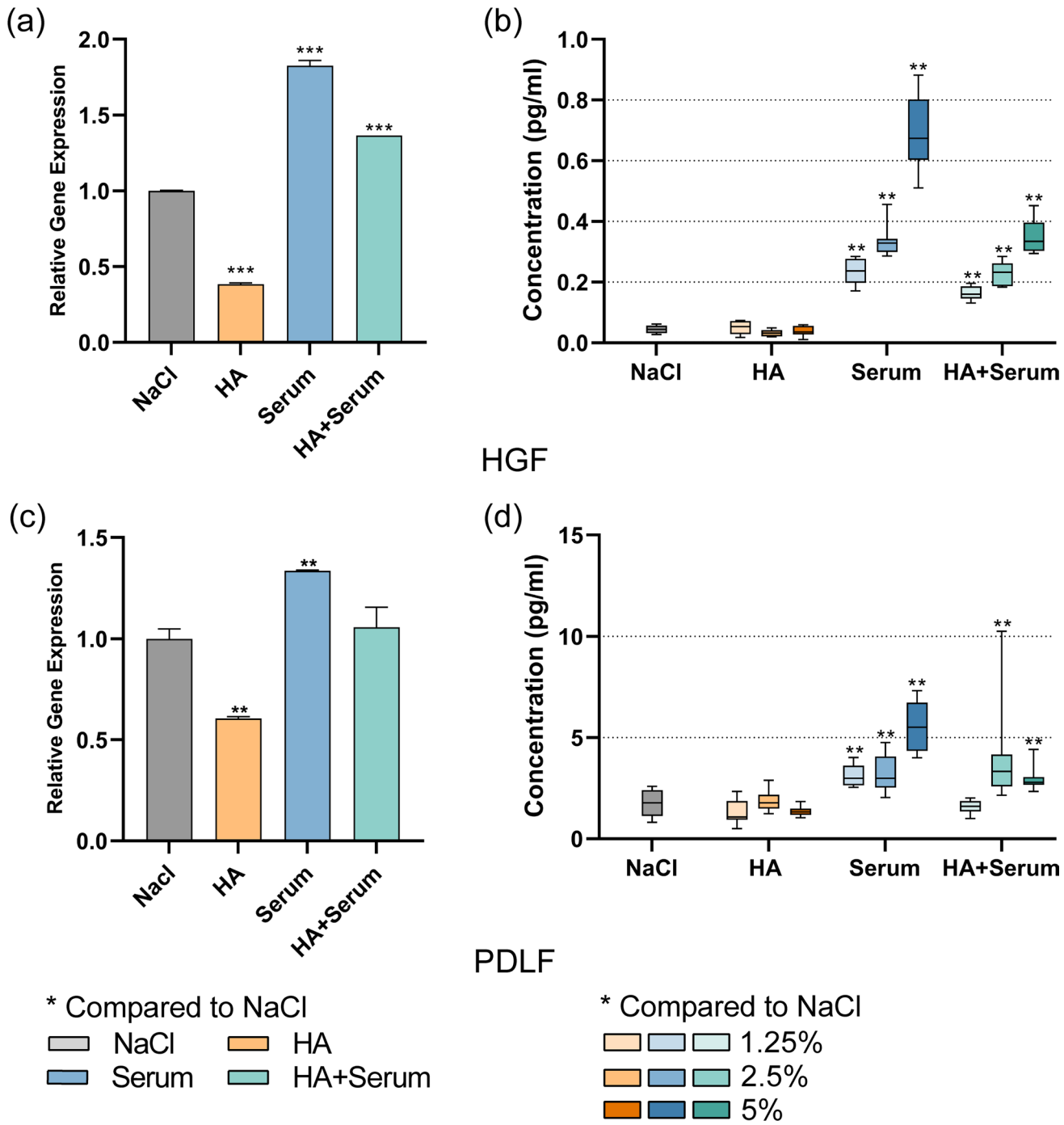

Fig. 4 Influence of hyaluronic acid (cHA), human serum (HS), and hyaluronic acid/human serum (cHA/HS) on mRNA expression (**a**, **c**) and protein level (**b**, **d**) of interleukin-8 in **a**, **b** gingival fibroblasts, and **c**, **d** periodontal ligament fibroblasts. mRNA expression: mean ± SD, protein: median incl. 25 and 75 percentiles. $*p < 0.05$, $**p < 0.01$ vs. control

at the same time, positively affect the healing/regenerative potential of the host cells. Gingival crevicular fluid does contain not only serum, but also, besides the presence of serum proteins, a number of other markers involved in the innate and acquired immune response [29].

The results of the present study have shown that both cHA and HS interfered with initial biofilm formation; however, they did not affect adhesion of PDLF to dentin. A further finding was that HS increased the expression of IL-8 by periodontal fibroblasts which was partially downregulated by cHA.

In biofilm formation, bacterial counts were only minorly affected. The strongest effect occurred at 4 h when the surface was coated both with cHA and HS. Interestingly, the results on biofilm quantity were more remarkable. Initially, cHA increased the biofilm quantity since it was probably incorporated in the matrix of the multi-species biofilm. However, at 24 h, the quantity was reduced when the surface was coated with cHA and/or HS. Recently, it was reported that HS inhibited biofilm formation of pathogens including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* although it did not affect planktonic growth, but the addition of an antibiofilm compound could reverse this effect [30]. In the present study, an increased inhibitory effect of cHA on HS was found. Interactions of HS albumin with HA can enlarge the binding ability of HA, as some positive divalent cations, e.g., Ca^{2+}, increase the affinity between them and contribute to lubrication [31]. Reported results on HA influence on biofilm formation are controversial. When adding HA on single-species biofilms of respiratory pathogens continuously less biofilm was quantified, the finding was discussed as a disaggregation of the matrix [32]. Also linking a polymethacrylate surface with HA reduced the adhesion of *Staphylococcus epidermidis* [33]. But using HA synthesized by *Streptococcus equi* promoted biofilm formation of *Streptococcus pneumonia* [34]. Regarding bacteria being associated with periodontal disease, recently a decrease by 60% (0.4 log10) of viable counts of *P. gingivalis* after 72 h of biofilm formation was mentioned [35]. An interesting approach seems to be to supplement HA gels with antimicrobials, for example, a HA formulation releasing oxygen reduced *P. gingivalis* growth [36].

In the present study, no clear effect of cHA or/and HS on fibroblast adhesion to dentin surfaces was found. This finding is in line with the results of a recent study [37] where cHA did not change the numbers of adhered PDLF to dentin surfaces. However, it has to be kept in mind that the analysis was made after 8 h and not after 72 h as in our study. Studies on non-cross-linked HA (ncHA) showed an inhibition of fibroblast adhesion and proliferation [33, 38]. cHA is less water soluble and promotes more cell proliferation than ncHA [39]. However, PDLF cultured on plastic surface showed an increase of fibroblast counts (proliferation) by about 20–30% by high-molecular weight HA, irrespective of whether cross-linked or not [40]. Both HA (cHA and ncHA) formulations have a high biocompatibility; in several studies, no negative effect on fibroblast viability was found [14, 40].

As the periodontal fibroblasts also function as immune cells [13], the IL-8 expression was analyzed. IL-8 is one of the most abundant proinflammatory cytokines in the oral cavity; in periodontal disease, it is produced by fibroblasts, epithelial cells, keratinocytes, and macrophages in response to the inflammatory reaction caused by bacteria and their components [41]. HS increased the expression of IL-8 which might be confirmatory to other studies. Serum amyloid A induces the expression of IL-8 in human gingival fibroblasts [42]. HS and its component serum albumin increased the expression of IL-8 by epithelial cells, also after challenging *P. gingivalis* and its obvious ability to degrade IL-8 [43]. IL-8 is a chemoattractant for neutrophils to the site of infection [44]. A positive role of neutrophils in battling the non-balanced microbiota can be assumed; however, neutrophils are also associated with tissue damage [45]. A downregulation of mRNA expression by cHA was found in that study; cHA decreased but did not block IL-8 expression induced by serum. This finding may support a beneficial role in the resolution of inflammation in periodontal therapy. Chen et al. showed that gingival fibroblasts after pretreatment with high-molecular weight HA and thereafter with *P. gingivalis* released less IL-8 in comparison with HA of lower molecular weight [46]. In the inflammatory model of interstitial cystitis, HA showed potent inhibition of IL-8 release [47]. IL-8 binds to HA, the binding is depending on the sulfation degree and the presence of metallic ions [48].

HA is triggering via the receptors RHAMM, CD44, and the intracellular adhesion molecule (ICAM)-1 [2]. Following injury, there is an increased expression of hyaluronic acid receptor genes in the initial stage of inflammation which promotes fibroblasts migration [49]. The focus on the present study was on RHAMM and CD44. The receptors are expressed both by the PDLF and the GF. Expression of CD44 was not affected neither by HS nor by cHA. CD44 is involved in wound healing thereby decreasing inflammatory response [50]. In case of RHAMM, the two types of fibroblasts responded differently to the stimuli. PDLF RHAMM expression did not significantly differ, whereas HS and/or cHA promoted the RHAMM expression in gingival fibroblasts. RHAMM expression is known to be stimulated by low-molecular weight HA. Signaling via the receptor leads to wound closure and resolution of inflammation [51]. The observed increase of RHAMM expression by HS may, at least partly, be responsible for cHA exerting its activity.

Animal models and in vitro research shed light to the role of HA in periodontal regeneration. A study on two wall intrabony defects in dogs which were treated with cHA and a collagen matrix highlighted the role of cHA in promoting periodontal wound healing/regeneration [15]. In diabetic rats, adding cHA to a collagen membrane prevented its premature degradation [52]. In vitro both cHA and ncHA increased early osteogenic differentiation of primary PDL fibroblasts [40]. Both preparations induced proliferation and migration of the fibroblasts and upregulation of genes involved in wound healing and regeneration [14]. In palatal but not in gingival fibroblasts, expression of matrix-metalloproteinases was induced, a finding of relevance when applying palatal transplants in periodontal surgery [14]. Further,

the proliferation of mesenchymal stromal and osteogenic progenitor cells was increased by cHA and ncHA [53].

In summary, the present study analyzed the role of cHA in the serum-rich environment of a periodontal pocket. It was shown that the serum did not negatively affect the activity of cHA against periodontal biofilm and on periodontal fibroblasts which, in turn, may support the application of cHA in non-surgical periodontal therapy. However, the present study has also some limitations. First of all, this is an in vitro study which did not consider the complexity of the periodontal region with a plethora of cells interacting with each other. Although here not tested, we assume that similar results concerning the effect of serum and cHA can be expected also for epithelial cells and alveolar bone cells. Second, an interaction of the periodontal biofilm with host cells (e.g., fibroblasts and monocytic cells) was not studied, and third, only one HA formulation (i.e., cHA) was used. Nevertheless, the results of the study encourage further in vitro research including other cell types and interactions with a periodontal biofilm and other HA formulations.

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Author contribution XZ performed the experiments; LvonW and GZ were involved in cell counting; SE, AS, and AS designed the study; SE supervised and designed the experiments; XZ, SE, and AS wrote the first draft of the manuscript; AS critically revised the manuscript.

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Declarations

Competing interests The authors declare no competing interests.

Ethics approval and consent to participate Human gingival fibroblasts (GF) and human periodontal ligament fibroblasts (PDLF) were obtained from extracted teeth whose owner had been informed and given written agreement. As these biomaterials were irreversibly anonymized, no approval of the local ethical committee (KEK) was needed according to the respective guidelines.

Conflict of interest The authors declare no competing interests.

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In-vitro effects of different hyaluronic acids on periodontal biofilm-immune cell interaction

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Introduction: Recent studies have demonstrated a positive role of hyaluronic acid (HA) on periodontal clinical outcomes. This *in-vitro* study aimed to investigate the impact of four different HAs on interactions between periodontal biofilm and immune cells.

Methods: The four HAs included: high-molecular-weight HA (HHA, non-cross-linked), low-molecular-weight HA (LHA), oligomers HA (OHA), and cross-linked high-molecular-weight HA (CHA). Serial experiments were conducted to verify the influence of HAs on: (i) 12-species periodontal biofilm (formation and pre-existing); (ii) expression of inflammatory cytokines and HA receptors in monocytic (MONO-MAC-6) cells and periodontal ligament fibroblasts (PDLF) with or without exposure to periodontal biofilms; (iii) generation of reactive oxygen species (ROS) in MONO-MAC-6 cells and PDLF with presence of biofilm and HA.

Results: The results indicated that HHA and CHA reduced the bacterial counts in a newly formed (4-h) biofilm and in a pre-existing five-day-old biofilm. Without biofilm challenge, OHA triggered inflammatory reaction by increasing IL-1β and IL-10 levels in MONO-MAC cells and IL-8 in PDLF in a time-dependent manner, whereas CHA suppressed this response by inhibiting the expression of IL-10 in MONO-MAC cells and IL-8 in PDLF. Under biofilm challenge, HA decreased the expression of IL-1β (most decreasing HHA) and increased IL-10 levels in MONO-MAC-6 cells in a molecular weight dependent manner (most increasing CHA). The interaction between HA and both cells may occur via ICAM-1 receptor. Biofilm stimulus increased ROS levels in MONO-MAC-6 cells and PDLF, but only HHA slightly suppressed the high generation of ROS induced by biofilm stimulation in both cells.

Conclusion: Overall, these results indicate that OHA induces inflammation, while HHA and CHA exhibit anti-biofilm, primarily anti-inflammatory, and antioxidant properties in the periodontal environment.

KEYWORDS

hyaluronic acid, periodontitis, periodontal therapy, macrophages, periodontal ligament fibroblasts, anti-biofilm activity, anti-inflammation, antioxidation

1 Introduction

Hyaluronic acid (HA), also known as hyaluronan, is a natural polysaccharide molecule, first isolated by Karl Meyer and John Palmer in 1934, and it is the only type of glycosaminoglycans (GAGs) that is not sulphated (Meyer and Palmer, 1934). HA consists of repeating disaccharide units of N-acetyl-glucosamine and glucuronic acid. Depending on the number of composed units, it has a wide range of molecular weight (MW), from a few dimers to millions of Daltons (Da) (Fraser et al., 1997). HA can be divided into high-MW HA (>1000 kDa) and low-MW HA (<500 kDa). Low-MW HA can further fragment into shorter oligomers (Jiang et al., 2011). Furthermore, HA exists in many formulations based on chemical modification of interunit chemical groups, including cross-linked and non-cross-linked (Choi et al., 2015).

HA occurs in many parts of the human body, including the skin, skeleton, joints (Fraser et al., 1997), and oral tissues (Martins et al., 2003). It is one of the primary components of the extracellular matrix (ECM) that regulates normal tissue integrity and development. Through interaction with cell surface receptors, HA plays important roles in inflammation and regeneration (Garantziotis and Savani, 2019). Some well-known HA receptors include homing cell adhesion molecule CD44, toll-like receptors 2/4 (TLR2/4), receptor for hyaluronan-mediated motility (RHAMM), and intercellular adhesion molecule-1 (ICAM-1), involved in cell growth, adhesion, motility together with their downstream signaling pathways (Jiang et al., 2011). HA has been suggested to have diverse biological functions depending on its MW and composition. For instance, HA fragments are believed to be pro-inflammatory and promote angiogenesis in many diseases, whereas high molecular weight HA (HHA) is considered to be more anti-oxidant, anti-inflammatory and anti-angiogenic (Litwiniuk et al., 2016). Due to these properties, HA is now widely used in tissue regeneration as well as in anti-aging and anti-inflammatory agents (Vasvani et al., 2020). In dentistry, HA has been reported to have a beneficial effect on clinical outcomes as an adjunct to non-surgical and surgical periodontal treatment (Bertl et al., 2015; Eliezer et al., 2019).

Periodontitis is characterized as a microbial-associated, host-mediated inflammation leading to periodontal attachment loss, tissue destruction, and ultimately tooth loss (Kinane et al., 2017). Keystone pathogens such as *Porphyromonas gingivalis* (Hajishengallis et al., 2012) along with accessory pathogens initially over-activate the inflammatory response and cause periodontal tissue destruction (Hajishengallis, 2014). However, the microbiome and host inflammatory response, involving networks of cytokines, chemokines and growth factors, are in bidirectional imbalance during disease propagation, and their interaction determines disease regression (Curtis et al., 2020). One of the hallmarks of periodontitis is the complex cell infiltration including polymorphonuclear neutrophils (PMNs), granulocytes, monocytes, and lymphocyte infiltration (Kinane et al., 2017). Macrophages contribute significantly to tissue homeostasis and defense by polarizing into M1 and M2 phenotypes. M1-type cells are activated by interferon gamma (IFN-γ) and lipopolysaccharides (LPS) and secrete pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6; whereas M2-type cells respond to IL-4 and IL-13 and participate in resolution of inflammation as evidenced by high levels of IL-10 and transforming growth factor-β (TGF-β) (Sun et al., 2021). Periodontal ligament fibroblasts (PDLF) are the most abundant cells in the periodontal ligament, which anchors teeth to alveolar bone for support and protection (Beertsen et al., 1997). Aside from its crucial role in periodontal tissue remodeling and homeostasis, PDLF also performs an immunomodulatory role during periodontitis progression by generating immune mediators such as chemokine IL-8. PDLF overexpression of proinflammatory cytokines may lead to amplification of the local inflammation by constantly triggering the immune response (El-Awady et al., 2010).

Clinical studies have demonstrated that the adjunctive application of high molecular weight (MW) HA after instrumentation has favorable effects on clinical outcomes and prevents the recolonization of periodontal pathogens (Eick et al., 2013). *In vitro* findings also revealed that high-MW HA (both cross-linked and non-cross-linked) enhanced the surface roughness of dentine discs with a high survival rate and spreading of PDLF (Mueller et al., 2017). Cells involved in periodontal tissue regeneration, such as PDLF, palatal and gingival fibroblasts, showed high cell viability and boosted proliferation when exposed to high-MW HA, suggesting high biocompatibility of HA for periodontal use (Fujioka-Kobayashi et al., 2017; Asparuhova et al., 2019).

However, considering the diverse functions associated with different molecular weights (MW) of HA, the specific impact of varying MWs of HA on the periodontal environment, as well as its potential mechanisms, remains unclear. The aim of this *in-vitro* study was to investigate the potential effects of different MWs of HA, as well as different formulations (cross-linked vs. non-cross-linked), on interactions between periodontal biofilm and immune cells.

2 Methods

2.1 HA preparation

Four different HA were used in this study: three non-cross-linked HAs (Bloomage Biotech, Jinan, China) of varying MW: 6 kDa HA oligomers (OHA), low-MW 400 kDa HA (LHA), high-MW 1000 kDa HA (HHA) and one cross-linked high-MW 1000 kDa HA (CHA, Regedent AG, Zurich, Switzerland) containing 18 mg/ml HA.

A concentration of 4 mg/ml HA was used in all cell experiments. In biofilm experiments, for the 4-h biofilm formation, concentrations of 2 and 8 mg/ml HA were used, and in the case of 5-day pre-cultured biofilm, 18 mg/ml HA were applied.

2.2 Microorganisms and cultivation

Twelve bacterial species of bacteria were included in the biofilm experiments:

1. *Streptococcus gordonii* ATCC 10558
2. *Actinomyces naeslundii* ATCC 12104
3. *Fusobacterium nucleatum* ATCC 25586
4. *Campylobacter rectus* ATCC 33238
5. *Parvimonas micra* ATCC 33270
6. *Eikenella corrodens* ATCC 23834
7. *Prevotella intermedia* ATCC 2561
8. *Capnocytophaga gingivalis* ATCC 33624
9. *Porphyromonas gingivalis* ATCC 33277
10. *Tannerella forsythia* ATCC 43037
11. *Filifactor alocis* ATCC 33099
12. *Treponema denticola* ATCC 35405

Except for *T. denticola* [cultured in mycoplasma broth (BD, Franklin Lake, NJ)], the other 11 strains were maintained on Schaedler agar plates (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood, in the case of *T. forsythia* additionally with 10 mg/l N-acetylmuramic acid. All strains were cultured at 37°C in the respective atmosphere: *S. gordonii* and *A. naeslundii* with 10% CO_2, and the others in an anaerobic incubator.

2.3 Activity on periodontal bacteria and biofilm

2.3.1 Determination of minimum inhibitory concentration

The MIC values of different HAs were determined against the above-mentioned bacterial species except for *T. denticola* ATCC 35405. Two-fold dilution series ranging from 0 to 20 mg/ml were prepared for different HAs. The microorganisms were then suspended in a two-fold concentrated Wilkins-Chalgren broth (Oxoid) and combined with varying concentrations of the different HAs at a 1:1 ratio. After incubating anaerobes for 24 h and aerobes for 18 h, the MIC values were determined as the lowest concentration with visible growth inhibition.

2.3.2 Biofilm assays

Two aspects of the 12-species periodontal biofilm were explored: (1) influence on biofilm formation and (2) influence on pre-existing biofilm.

(1) Influence on biofilm formation: wells of a 48-well plate were covered with 25 µl/well 1.5% bovine serum albumin (BSA)/0.67% mucin solution at room temperature for 1 h to generate a proteinaceous surface layer. Then, 50 µl/well of the different HA solutions (2 mg/ml and 8 mg/ml) was used), were added for another 30 min incubation. Thereafter, microbial suspension mixed with nutrient broth (Wilkins-Chalgren broth) in a volume ratio of 1:9 was added, 450 µl per well, meaning the final concentration of HA were 0.2 and 0.8 mg/ml respectively. Subsequently, the plate was incubated anaerobically at 37°C. The microbial suspension consisted of one part *S. gordonii*, two parts *A. naeslundii*, eight parts *T. denticola*, and four parts the other nine species, all of which were suspended in 0.9% NaCl at McFarland 4. After 4 h, the biofilms were scraped from the well surface after careful washing and resuspended in 0.9% NaCl. The biofilm suspension was serially diluted and sub-cultured on agar plates for colony forming units (CFU) assessment after one week of anaerobic incubation.

(2) Influence on pre-existing biofilm: wells of the 48-well plate were coated with protein solution as described above, and 450 µl/well of microbial suspension mixed with Wilkins–Chalgren broth (volume: 1:9, as stated above) was added. The plates were then cultured in an anaerobic incubator for 5 days, at day 3, the medium was replaced by fresh one supplemented with *P. gingivalis*, *T. forsythia*, and *T. denticola*. At day 5, the nutrient broth was carefully removed, and the biofilm was gently washed. Then, 50 µl//well HAs at 18 mg/ml (according to the concentration of CHA) was added to the biofilm for 1 min, followed by 450 µl/well Wilkins-Chalgren broth. After 1 h of anaerobic incubation, the biofilms were scraped from the well surface and resuspended in 0.9% NaCl into aliquots: one part for CFU, one part for biofilm mass quantification via crystal violet staining (Merritt et al., 2005), and one part for biofilm metabolic activity via Alamar blue staining as previously described (Pettit et al., 2005).

2.3.3 Live/dead staining

Based on the significant results of biofilm formation in the HHA and CHA groups, the 4-h biofilms treated with HHA and CHA (8 mg/ml) were stained using LIVE/DEAD® BacLight Bacterial Viability Kits (Molecular Probes, Life technologies, USA) according to the manufacturer's instructions. The images were then taken using a Zeiss LSM 710 confocal microscope (Carl Zeiss) with oil immersion in two fluorescence channels (green and red). Green staining indicates live cells while red staining indicates dead cells. *Imarisviewer* software (Bitplane, *IMARIS* 10.0.0) was used for additional visualization.

2.4 Cell culture

The human monocytic cell line MONO-MAC-6 obtained from DMSZ (Braunschweig, Germany) was cultivated in RPMI 1640 medium supplemented with 10% double-heat-inactivated fetal bovine serum (FBS), 1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 µg/ml human insulin (Invitrogen; Carlsbad, CA, USA).

Human PDLF were isolated from freshly extracted and donated premolar teeth from systemically healthy young adults undergoing orthodontic therapy. The individuals had been informed about the use of teeth for research purposes and completed a written consent form. According to the criteria of the Cantonal Ethical Committee (KEK), there is no need for additional approval if the biomaterials are classified as "irreversibly anonymized". The primary cell culture

procedure was applied as previously described (Lin et al., 2020). Briefly, tissues from the mid-third of the teeth were washed three times with PBS before being minced into 1-mm^3 cubes. The cubes were then grown on average in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen; Carlsbad, CA, USA) supplemented with 10% FBS supplemented with an antibiotic-antimycotic solution (Gibco, Thermo Fischer, MA, USA) containing 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml of amphotericin B as the final concentration. Cells from at least three donors were maintained.

Before being exposed to test substances, all cell strains were grown at 37°C with 5% CO_2 and starved overnight in 0.5% FBS/DMEM or 0.5% FBS/RPMI 1640 being also the "regular medium" in the experiments.

2.4.1 MTT assay

The cell viability was assessed using the MTT assay (Mosmann, 1983). PDLF were seeded in 96-well-plates at a density of $1x10^5$ cells per well and allowed to reach confluency for at least 24 hours. On the second day, the cells were gently washed twice with PBS, followed by a change to 100 µl/well HAs-medium (different HAs with a concentration ranging from 0 to 4 mg/ml in 0.5% FBS-DMEM). In the case of MONO-MAC-6 cells the cells were resuspended in HAs-medium (HAs in 0.5% FBS-RPMI). After 4 h treatment in HAs-medium, 10 µl/well MTT solution (final concentration, 0.5 mg/ml) was added for additional 2 h-incubation. The dark blue formazan crystals formed within viable cells were solubilized using lysis buffer (20 µl of 3% SDS and 100 of acid-propanol) and well mixed before measuring absorbance at 570 nm relative to the reference wavelength of 630 nm with a microplate reader (Agilent, CA, USA). The data was presented as a percentage (%) of control (untreated cells).

2.4.2 Release of inflammatory cytokines and HA receptors in MONO-MAC-6 cells and PDLF

In general, MONO-MAC-6 cells and PDLF were divided into two groups: (a) biofilm stimulated group (BS group): cultivated with different HAs-media under biofilm stimulations; (b) non-stimulated group (NS group): only HAs-medium. Quantitative PCR (qPCR) and ELISA were used to assess the inflammatory cytokine expression levels between the BS and NS groups, while qPCR was used to detect HA receptors.

First, a 48-h periodontal biofilm was created as previously indicated. For MONO-MAC-6 cells in BS groups, cells in HAs-medium (4 mg/ml different HAs in regular medium) were seeded into the wells with the formed biofilms after removing the microbial nutrient medium and properly washing. The cells in the NS group were seeded directly into the wells and cultured under the same conditions. For PDLF, the 48-h biofilms were harvested, adjusted to McFarland 4 and exposed to ultrasonication for 20 min and then centrifuged at 4000 g for 5 min to extract the biofilm supernatant. PDLF were seeded into wells to generate a monolayer for 24 h. On the second day, the medium was exchanged to HAs-medium (4 mg/ml), with or without 10% biofilm supernatant.

For ELISA, the cell suspensions were collected after certain incubation times (2 h, 4 h) and centrifuged at 8000 g before supernatants were obtained and stored at -80°C. The IL-1β and IL-10 protein levels were measured in the MONO-MAC-6 cells supernatants, and IL-8 in the PDLF supernatants using ELISA kits (R&D Systems Europe Ltd., Abingdon, UK) as instructed. For the mRNA expression, total RNA was extracted from both cells after 2 h of incubation using the innuPREP RNA Mini Kit 2.0 (Analytic Jena GmbH, Germany). The GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) was then used to reverse cDNA from 1000 ng RNA. GoTaq® qPCR Master Mix (Promega) was used along with the QuantStudio 3 RT-PCR System (Thermo Fischer, Waltham, MA, USA) to perform qPCR. GAPDH was used to normalize gene expression, and the $2^{-\triangle\triangle CT}$ method was used to assess the relative expression of the respective gene (Livak and Schmittgen, 2001). The IL1B and IL10 mRNA expression levels were measured in MONO-MAC-6 cells, and IL8 in PDLF; the HA receptor genes (CD44, RHAMM, TLR2, TLR4, and ICAM1) expressions were determined in both cells; the primers are indicated in Table 1.

2.4.3 Reactive oxygen species assay

The MONO-MAC-6 cells or PDLF were incubated for 1 h in HAs-medium (4 mg/ml) or regular medium with or without biofilm stimulation before total ROS generation was quantified. The ROS assay was carried out using the Cellular ROS assay kit (Red Fluorescence) (Abcam, Cambridge, UK) following the manufacture's instruction with a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, USA). The wavelengths of excitation and emission were 520 nm and 605 nm, respectively.

2.5 Statistical analysis

All experiments were repeated in at least two independent experiments with each quadruplicate. The data was displayed as mean and standard deviation (SD), and a log10 transformation was performed for CFU analysis.

The graphs were created with GraphPad Prism 9 (Graphpad Software, Bosten, MA, USA). SPSS 28.0 (IBM, Chicago, IL, USA) was used for statistical analysis. Following a Shapiro-Wilk test for normality, the one-way analysis of variance (one-way ANOVA) with *post-hoc* Tukey was conducted. The focus was on comparing the respective HA group with the control group and comparing the CHA with the HHA group. A *p*-value below 0.05 was considered statistically significant.

3 Results

3.1 Periodontal bacteria and biofilm

According to the MIC test, a concentration up to 10 mg/mL did not impede visible bacterial growth of all tested bacterial species.

TABLE 1 Primer sequences used for qPCR.

Gene	Forward/ Reverse primers	Primer sequences 5′-3′	References
IL8	F	GAG AGT GAT TGA GAG GTG GAC CAC	(Parisi et al., 2022)
	R	CAC AAC CCT CTG CAC CCA GTT T	
IL1B	F	TAC GAA TCT CCG ACC ACC ACT ACA G	
	R	TGG AGG TGG AGA GCT TTC AGT TCA TAT G	(Fernandez-Botran and Vetvicka, 2000)
IL10	F	GCC TAA CAT GCT TCG AGA TC	
	R	CTC ATG GCT TTG TAG ATG CC	(Chhabra et al., 2008)
TLR2	F	GGG TCA TCA TCA GCC TCT CC	
	R	AGG TCA CTG TTG CTA ATG TAG GTG	(Sabroe et al., 2002)
TLR4	F	CAG AGT TGC TTT CAA TGG CAT C	
	R	AGA CTG TAA TCA AGA ACC TGG AGG	(Sabroe et al., 2002)
ICAM1	F	AGC GGC TGA CGT GTG CAG TAA T	
	R	TCT GAG ACC TCT GGC TTC GTC A	(Ma et al., 2021)
CD44	F	GAC CTC TGC AAG GCT TTC AAT A	# M59040.1
	R	CAA AGG CAT TGG GCA GGT CT	
RHAMM	F	AGG ACC AGT ATC CTT TCA GAA ATC	# BC017793.1
	R	AGT GCA GCA TTT AGC CTT GC	
GAPDH	F	GAC AGT CAG CCG CAT CTT CT	(Shen et al., 2010)
	R	TTA AAA GCA GCC CTG GTG AC	

Two aspects of periodontal biofilm were investigated in terms of the potential effects of different MW as well as of the cross-linked structure of HA: biofilm formation and biofilm destruction.

3.1.1 Early biofilm formation

To assess the impact on biofilm formation, surfaces were coated with HA solutions (each 2 mg/ml and 8 mg/ml) prior adding the microbial suspension for biofilm formation.

As demonstrated in Figure 1A, only the 8 mg/mL HHA and CHA solution resulted in a significant reduction (-0.51 log10 by HHA and -0.78 log10 by CHA) of CFU compared to the control (both $p<0.001$). Comparing HHA and CHA, lower CFU counts were always found after the coating of 2 and 8 mg/ml CHA vs. the respective concentration of HHA (by -0.33 log10, $p=0.009$ and by -0.27 log10, $p=0.028$).

Considering the reducing effect of 8 mg/ml HHA and CHA, live/dead staining was performed on 4 h-biofilms where the surfaces were coated with 8 mg/ml of HHA and CHA. The confocal images in Figure 1B indicated that both HHA and CHA groups resulted in lower density and thickness of the 4-h biofilms compared to the control group, with CHA having the lowest values.

3.1.2 Mature biofilm destruction

To evaluate the effect of HA on mature biofilm, 18 mg/ml HAs, simulating the concentration of the commercial product was applied to the pre-existing five-day biofilm for 1 min.

As shown in Figure 2A, HAs did not significantly influence the CFU counts in the 5 d-biofilm. Regarding biofilm mass, only HHA and CHA induced a significant reduction (each $p<0.001$, Figure 2B). CHA seemed to reduce more biofilm mass than HHA, but there was no statistical significance. The metabolic activity of the biofilm increased in LHA and HHA group (each $p<0.001$, Figure 2C). The metabolic activity was less in CHA than in HHA group ($p<0.001$), Figure 2C.

3.2 Immune interaction between MONO-MAC-6 cells and biofilm stimulation

The effect of different HAs on the inflammatory response of a monocytic cell line (MONO-MAC-6) with or without a periodontal biofilm challenge was investigated in four aspects: relative expression of inflammatory cytokines, expression of HA receptors, and oxidative stress.

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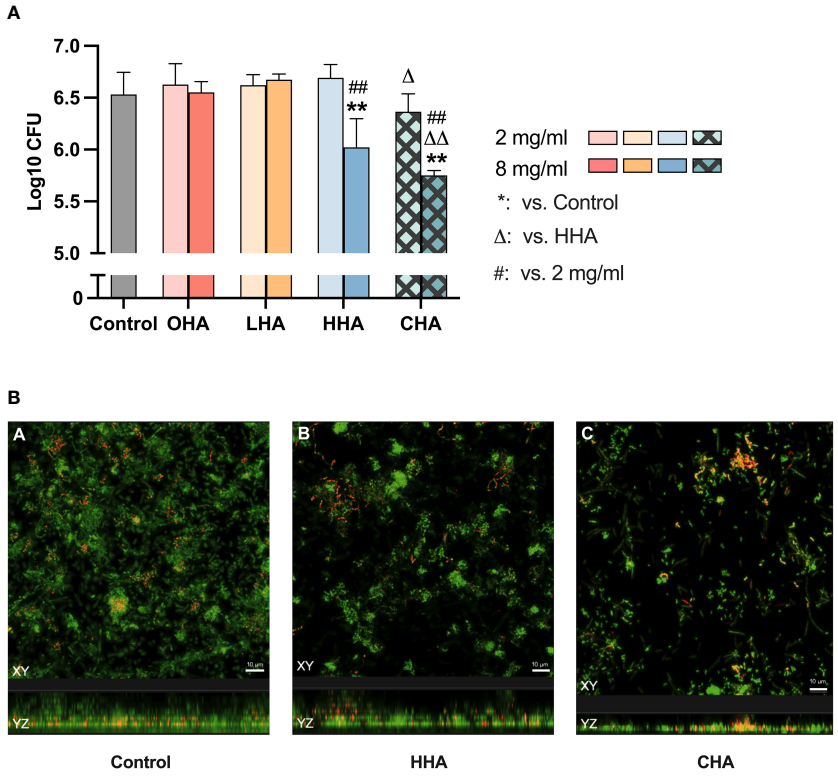

FIGURE 1
Impact of coating with 2 mg/ml and 8 mg/ml hyaluronic acid (HA) (OHA: 6 kDa, non-cross-linked; LHA: 400 kDa, non-cross-linked; HHA: 1000 kDa, non-cross-linked; CHA: 1000 kDa, cross-linked) on early (4 h) periodontal biofilm formation: **(A)** colony forming units (CFU; mean and SD). ** $p<0.01$ vs. control group, △/△△ $p<0.05/0.01$ vs. respective concentration HHA. **(B)** Confocal laser scanning microscopy images using live/dead staining assay (green live cells, red dead cells). **(A)** Control biofilm (ii) Biofilm treated with 8 mg/ml HHA. (iii) Biofilm treated with 8 mg/ml CHA. Scale bar = 10 µm.

To confirm sufficient cell viability in the experimental conditions, the MTT test was performed on MONO-MAC-6 cells being exposed to 4 mg/ml HA for 4 h. The viability of MONO-MAC-6 cells did not remarkably decrease, it remained more than 75% throughout (data not shown in detail).

3.2.1 Expression of IL-1β and IL-10 in MONO-MAC-6 cells

To assess inflammatory cytokine expression, we investigated the expression of both IL-1β and IL-10 at the protein and mRNA levels in MONO-MAC-6 cells. MONO-MAC-6 cells in HAs-medium (4 mg/ml) or without HA (control group) were exposed to periodontal biofilm (BS) or not (NS) for 2 h and 4 h for protein detection and 2 h for mRNA detection.

3.2.1.1 Protein expression

At the protein level, in the absence of HA, MONO-MAC-6 cells released more IL-1β (Figures 3A, B, both $p<0.001$ at 2 h and 4 h) and less IL-10 (Figures 3D, E, $p<0.001$ at both 2 h and 4 h) when challenged with periodontal biofilm for 2 h and 4 h, compared to those without biofilm.

In the NS group (Figures 3A, D), higher IL-1β and IL-10 levels were observed over time in the OHA group, with a significant difference noted at 2 h for IL-1β ($p<0.001$), and at 4 h for IL-10 ($p<0.001$). However, LHA slightly decreased the amount of IL-10 at both 2 and 4 h compared to the control ($p<0.001$ at 2 h). No significant changes were observed in MONO-MAC-6 release of IL-1β and IL-10 for the HHA group; the CHA group decreased the IL-10 levels at 2 h ($p<0.001$ vs. control, and $p<0.001$ vs. HHA) and at 4 h ($p=0.044$ vs. control).

In the BS group (Figure 3B), at 2 h, the level of IL-1β decreased in an MW-dependent manner with HHA having the lowest expression ($p=0.001$ for LHA, HHA and CHA groups vs. BS control). A similar trend was observed among the HA groups after 4 h, but only HHA reached statistical significance ($p=0.001$). Regarding IL-10 levels in the BS group (Figure 3E), HA increased the IL-10 expression in a MW-dependent behavior, with CHA had the highest increasing effect at both 2 h and 4 h, $p<0.001$ vs. control and $p=0.003$ vs. HHA.

3.2.1.2 mRNA expression

At the mRNA level (Figures 3C, F) indicated that biofilm stimulation primarily upregulated the gene expression of IL1B (333-fold change for IL1B, $p=0.029$) and by trend of IL10 (5.0-fold change, $p=0.108$). In the presence of HA, OHA significantly

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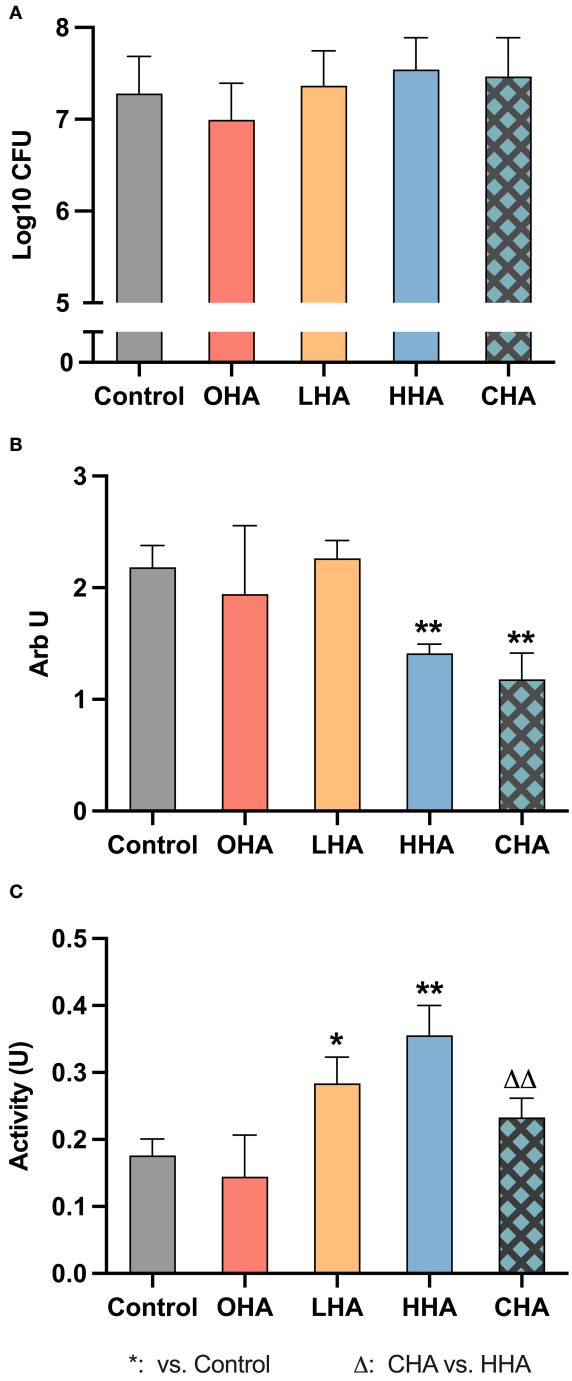

FIGURE 2
Impact of 18 mg/ml for 1 min hyaluronic acid (HA) (OHA: 6 kDa, non-cross-linked; LHA: 400 kDa, non-cross-linked; HHA: 1000 kDa, non-cross-linked; CHA: 1000 kDa, cross-linked) on the destruction of pre-formed five-day periodontal biofilm: **(A)** Colony forming units (CFU) counts, **(B)** quantity, and **(C)** metabolic activity The CFU values were subjected to a log10 transformation. The results are presented as Mean ± SD, */** $p<0.05$/$p<0.01$ vs. control group, △△ $p<0.01$ vs. HHA.

elevated the mRNA expression of both IL1B and IL-10 in NS groups by 266-fold change and 10.2-fold change respectively (both $p<0.001$ vs. NS control). In BS groups, HA did neither influence the IL1B expression nor the IL10 mRNA levels.

3.2.2 Expression of HA receptors in MONO-MAC-6 cells

The mRNA expression of five major HA receptor genes (CD44, RHAMM, TLR2, TLR4, and ICAM1) was investigated in MONO-MAC-6 cells. The cells were stimulated with or without biofilm and were treated with or without HA. Different HAs (4 mg/ml) for each mRNA expression assay were used, with an incubation time of 2 h. However, among the studied receptors, only ICAM1 was affected by the stimuli (Figure 4), the expression of the other four receptor genes (CD44, RHAMM, TLR2, and TLR4) were not influenced by either HA or biofilm stimulation (data not shown).

Biofilm stimulation increased ICAM1 mRNA expression in MONO-MAC-6 cells by 8.9-fold ($p=0.028$) without supplemented HA (Figure 4). In NS groups under HA conditions, OHA significantly increased ICAM1 mRNA expression by 9.5-fold change ($p<0.001$), while HHA slightly increased ICAM1 mRNA expression by 2.5-fold change ($p=0.011$). In terms of CHA, it did not cause an upregulation compared to control, the mRNA expression was lower than that induced by HHA ($p<0.006$).

In BS group, all HA groups decreased by trend ICAM1 mRNA (not statistically significant).

3.2.3 Oxidative stress in MONO-MAC-6 cells

The total ROS level was quantified to investigate whether the biofilm burden caused oxidative stress in MONO-MAC-6 cells, and to evaluate the effect of HA as potential antioxidant under these conditions. Medium without or containing 4 mg/mL of HAs was applied to the cells and those were exposed to the biofilm or not for 1 h, before subsequently the ROS level was measured.

According to Figure 5, biofilm stimulation significantly increased ROS levels compared to NS groups in the corresponding medium condition [$p<0.01$ in all groups (with and without HAs)]. In NS groups, the addition of HA to MONO-MAC-6 cells did not significantly affect the ROS levels ($p>0.05$ in all groups vs. control). In the BS groups, only the HHA treatment showed a statistically significant reduction in ROS vs. BS control ($p=0.039$).

3.3 Immune interaction between PDLF and biofilm stimulation

The inflammatory response of PDLF with and without biofilm lysate stimulation was investigated. As before several aspects were examined, including expression of inflammatory chemokine, HA receptors, and oxidative stress.

As before the cell viability was tested under the experimental conditions, it was always more than 75%.

3.3.1 Expression of IL-8 in PDLF

Protein and mRNA expressions of IL-8 were evaluated in PDLF. Cells were cultured in either regular medium or HAs-medium (4 mg/ml) and were exposed in part to biofilm supernatants. The release of IL-8 protein was measured after 2 h and 4 h, while the expression of IL-8 mRNA was measured after 2 h.

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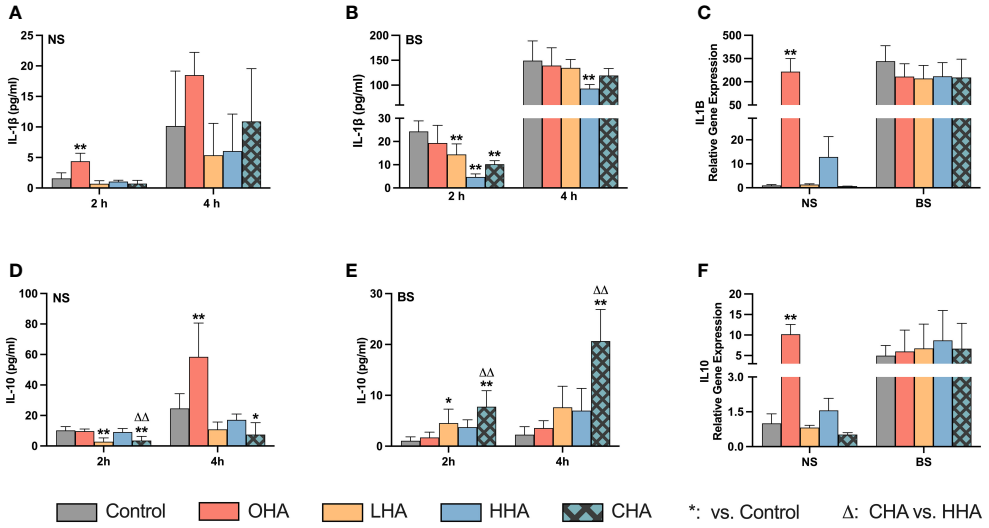

FIGURE 3
Effect of 4 mg/ml hyaluronic acid (HA) (OHA: 6 kDa, non-cross-linked; LHA: 400 kDa, non-cross-linked; HHA: 1000 kDa, non-cross-linked; CHA: 1000 kDa, cross-linked) on protein **(A, B, D, E)** and mRNA **(C, F)** expression of interleukin-1β [IL-1β **(A, B)**, ILB **(C)**] and interleukin-10 [IL-10 **(D, E)**, IL10 **(F)**] in a monocytic cell line (MONO-MAC-6 cells) with **(B, C, E, F)** or without **(A, D, E, F)** periodontal biofilm after 2 h and 4 h (only protein levels) of stimulation. NS, non-biofilm-stimulated; BS, biofilm-stimulated. Mean ± SD, */** p<0.05/p<0.01 vs. control, △△ p<0.01 vs. HHA group.

The release of IL-8 protein in PDLF increased over time in the corresponding medium in both NS and BS groups (Figures 6A, B). In the absence of HA, biofilm significantly increased IL-8 protein and mRNA expression vs. NS group (each p<0.001, Figures 6B, C).

In the NS groups, OHA increased IL-8 protein expression at 4 h (p<0.001). At the mRNA level, OHA and HHA increased IL8 mRNA expression with 3.7-fold and 3.6-fold change, respectively (both p<0.001). In contrast to HHA, CHA caused by trend a downregulation of IL8 mRNA expression to 0.1-fold of control (p=0.402) which is 0.03-fold of HHA (p<0.001).

In the BS group, there were no significant differences observed between HA groups and controls at the protein level after 2 h and 4 h (Figure 6B) and at the mRNA level after 2 h (Figure 6C). CHA slightly increased IL-8 protein level compared to HHA at 2 h (p=0.002). Also, the IL8 mRNA expression was increased by CHA by 1.5-fold compared to HHA (p=0.023).

3.3.2 Expression of HA receptors in PDLF

As in MONO-MAC-6-cells, HA receptor genes (CD44, RHAMM, TLR2, TLR4 and ICAM1) were also examined in PDLF after 2 h exposure to biofilm supernatants in medium without or with HAs (4 mg/ml).

As before for the MONO-MAC-6-cells, there were only differences between the groups found for the ICAM mRNA expression. Following only the ICAM results are presented (Figure 7).

The biofilm supernatant increased ICAM1 mRNA expression in PDLF, causing a 30.6-fold upregulation without HA. In the NS group, OHA increased ICAM1 mRNA expression by 5.6-fold

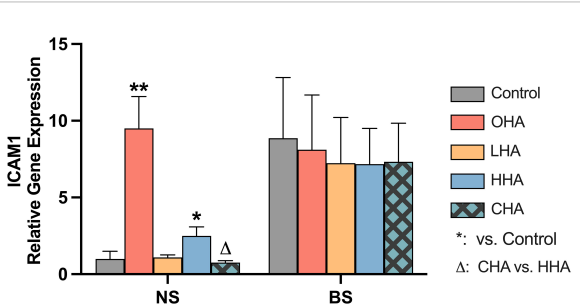

FIGURE 4
Effect of 4 mg/ml hyaluronic acid (HA) (OHA: 6 kDa, non-cross-linked; LHA: 400 kDa, non-cross-linked; HHA: 1000 kDa, non-cross-linked; CHA: 1000 kDa, cross-linked) on the mRNA expression levels of a HA receptor gene – ICAM1 in a monocytic cell line (MONO-MAC-6 cells) with or without biofilm after 2 h stimulation. NS, non-biofilm stimulated; BS, biofilm-stimulated. Mean ± SD, */** p<0.05/p<0.01 vs. control, △ p<0.05 vs. HHA group.

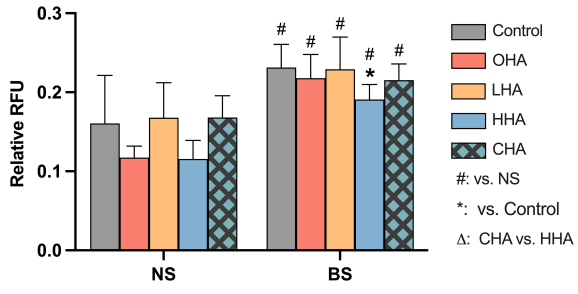

FIGURE 5
Effect of 4 mg/ml hyaluronic acid (HA) (OHA: 6 kDa, non-cross-linked; LHA: 400 kDa, non-cross-linked; HHA: 1000 kDa, non-cross-linked; CHA: 1000 kDa, cross-linked) on the total amount of reactive oxygen species (ROS) in a monocytic cell line (MONO-MAC-6 cells) with or without periodontal biofilm after 2 h of stimulation. NS, non-biofilm-stimulated; BS, biofilm-stimulated. Mean ± SD, */** p<0.05/p<0.01 vs. control, △ p<0.05 vs. HHA group and # p<0.05 vs. the corresponding group in NS cells.

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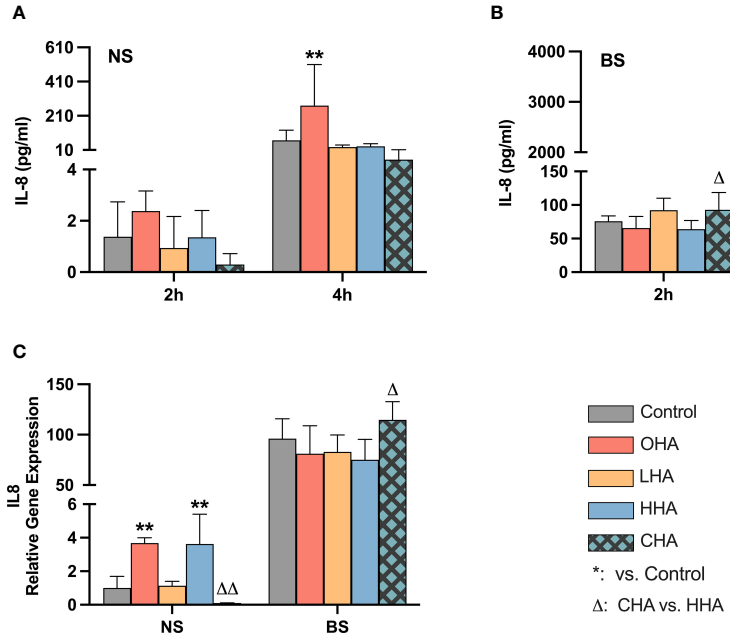

FIGURE 6
Effect of 4 mg/ml hyaluronic acid (HA) with different molecular weights (OHA: 6 kDa, non-cross-linked; LHA: 400 kDa, non-cross-linked; HHA: 1000 kDa, non-cross-linked; CHA: 1000 kDa, cross-linked) on protein **(A, B)** and mRNA **(C)** expression of interleukin-8 (IL-8, IL8) in periodontal ligament fibroblasts (PDLF) with **(B, C)** or without **(A, C)** periodontal biofilm after 2 h and 4 h (only protein levels) of stimulation. NS, non-biofilm-stimulated; BS, biofilm-stimulated. Mean ± SD, */** $p<0.05$/$p<0.01$ vs. control, △ $p<0.05$ vs. HHA group.

change ($p<0.001$). In the BS group, LHA, HHA, CHA seemed to decrease in ICAM1 mRNA expression, although it was not statistically significant.

3.3.3 Oxidative stress in PDLF

The total level of ROS was measured in PDLF after 1 h exposure to HA medium (4 mg/ml) and biofilm stimulation (Figure 8).

Biofilm stimulation significantly increased ROS generation in PDLF ($p<0.001$). In the NS group, there was no statistical significance observed among all groups. In the BS group, the addition of HA resulted in statistical significance only in the HHA group ($p=0.046$) in reducing the high level of ROS induced upon biofilm stimulation.

4 Discussion

The objective of this study was to investigate the potential effects of different molecular weights (MW) of HA, as well as two different formulations (cross-linked vs. non-cross-linked), on the interaction between periodontal biofilm and immune cells. This study is the first to report the potential anti-biofilm, anti-oxidative, and anti-

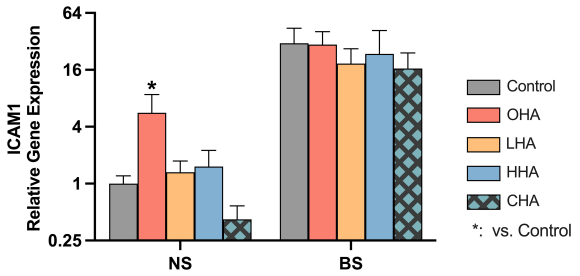

FIGURE 7
Effect of 4 mg/ml hyaluronic acid (HA) (OHA: 6 kDa, non-cross-linked; LHA: 400 kDa, non-cross-linked; HHA: 1000 kDa, non-cross-linked; CHA: 1000 kDa, cross-linked) on the mRNA expression levels of a HA receptor gene – ICAM1 in periodontal ligament fibroblasts (PDLF). with or without biofilm after 2 h stimulation. NS, non-biofilm-stimulated; BS, biofilm-stimulated, Mean ± SD, * $p<0.05$ vs. control.

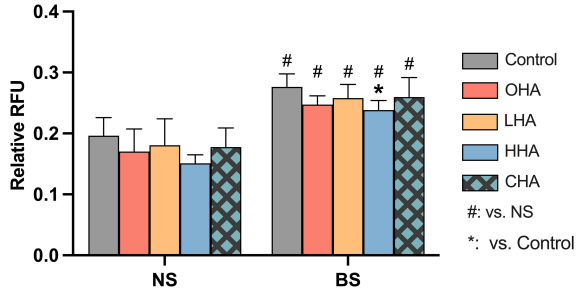

FIGURE 8
Effect of 4 mg/ml hyaluronic acid (HA) (OHA: 6 kDa, non-cross-linked; LHA: 400 kDa, non-cross-linked; HHA: 1000 kDa, non-cross-linked; CHA: 1000 kDa, cross-linked) on the total amount of reactive oxygen species (ROS) in periodontal ligament fibroblasts (PDLF) with or without periodontal biofilm after 1 h of stimulation. NS, non-stimulated; BS, biofilm-stimulated. Mean ± SD, * $p<0.05$ vs. control and # $p<0.05$ vs. the corresponding group in NS cells.

inflammatory properties of various HAs when directly challenged by a 12-species periodontal biofilm. The results underscore that HA influenced the periodontal biofilm itself and modulated the immune response of oral cells, with the effect depending on the MW and chemical modification (cross-linked or non-cross-linked).

Our primary objective was to investigate the effect of different HAs on the 12-species periodontal biofilms. None of the tested HAs (up to 10 mg/ml) clearly inhibited the growth of the included 11 oral species. Data on MIC values of HA alone on planktonic bacteria are scarce. However, higher concentrations of 40 mg/ml were found to be growth inhibitory against β-hemolytic streptococci, *Staphylococcus aureus*, *S. epidermidis* (Carlson et al., 2004). However, our results differ from those of other studies that determined an MIC of 4 mg/ml against *P. gingivalis* (Alharbi and Alshehri, 2022).

Conversely, the two types of high-MW HA inhibited the formation of the tested 12-species periodontal biofilm in a concentration-dependent manner, with CHA showing the strongest effect in the present study. This anti-biofilm formation ability of HAs aligns with the findings from other studies.

For example, HA inhibited single biofilm formation, demonstrating a more sensitive effect on the biofilm produced by *Staphylococcus aureus* than *Haemophilus influenzae* and *Moraxella catarrhalis* (Drago et al., 2014). In general, a hydrophilic and negatively charged surface prevents bacteria adhesion (Delaviz et al., 2015). In this study, the negatively charged HA may form a hydrophilic layer on the mucin-BSA-coated surface, which may prevent planktonic bacteria from attaching to the proteinaceous surface, thereby inhibiting the initial stage of biofilm formation (Hannig and Hannig, 2009; Fallacara et al., 2018). The extent of the formed hydrophilic layer is correlated with the MW size (Fallacara et al., 2018) which may explain that only the high-MW HAs (HHA and CHA) act inhibitory. Regarding the stronger effect of CHA, its cross-linked structure provides a network of layers that slows down diffusion and serves as a more stable barrier (De Boulle et al., 2013).

Another essential aspect is the impact of HA on destruction of a pre-existing biofilm. The analysis revealed that both HHA and CHA reduced the biomass. This effect could potentially be attributed to their ability to dissolve the biofilm matrix, as there were no changes observed in the CFU counts or reductions in the metabolic activity of the biofilm microorganisms. Similarly, Champion et al. demonstrated that high-MW HA affected the biomass but not the bacterial counts of already-formed *P. aeruginosa* biofilm (Champion et al., 2022). Investigating the potential destruction of biofilm matrix might be of interest in upcoming studies.

The *in-vitro* findings on the anti-biofilm effect of HA might support clinical data on oral bacteria, where applying high MW HA gel in the peri-implant sulcus for 45 days reduced the relative abundance of peri-implantitis-related microorganisms, particularly *Prevotella and Campylobacter* (Soriano-Lerma et al., 2020).

Periodontal disease is not solely caused by biofilm itself, it results also from the host immune response to the microbes (Cekici et al., 2014). In the dynamic development of periodontitis, macrophages play an essential role in immune regulation and phagocytosis by differentiating to different phenotypes (Sun et al., 2021). The M1 phenotype promotes killing of bacteria and increases inflammation by producing high levels of pro-inflammatory cytokines like IL-1β and TNF-α. In contrast, the M2 phenotype induces tissue regeneration by releasing anti-inflammatory cytokines like IL-10 and TGF-β (Orekhov et al., 2019). The M1/M2 ratio was found to be increased in periodontitis (Yu et al., 2016). This may be supported by the present *in-vitro* study with highly elevated levels of IL-1β and decreased levels of IL-10 in MONO-MAC-6 cells after biofilm stimulation.

The effects of HA on macrophages are closely related to its MW. As investigated with murine macrophages, regardless of the initial polarization state of macrophages, macrophages underwent phenotypic alterations based on MW of HA, which was a pro-inflammatory response for lower MW HA and oligos of HA (no more than 5 kDa) and a pro-resolving response for higher MW HA (3000 kDa), while the response to intermediate-MW HA (60 kDa – 800 kDa) was difficult to ascertain (Rayahin et al., 2015). In a similar study, high-MW HA (1500 kDa) caused a concentration-dependent reduction in IL-1β in murine macrophages stimulated with LPS, while low-MW HA (100 kDa and 500 kDa) caused an increase in IL-1β. But at both 500 kDa and 1500 kDa MW, HA increased IL-10 in LPS-stimulated macrophages (Lee et al., 2021). In the present study, we found that without biofilm stimulation, OHA induced both IL-1β and IL-10 in MONO-MAC-6 cells, while with biofilm stimulation, high-MW HA (HHA and CHA) decreased IL-1β and increased IL-10. Together with our findings, it suggests that OHA promotes inflammation whereas high-MW HA decreases inflammation induced by bacterial stimulus.

The contrasting response to inflammation in MONO-MAC-6 cells to the different molecular weights (MWs) of HA may correspond with varying affinities to the receptors, which subsequently affect the downstream signaling pathways (Yang et al., 2012). CD44, TLR2/4, RHAMM and LYE-1, ICAM-1 have been reported as the receptors of HA (Vasvani et al., 2020). However, except for ICAM-1, we did not find any difference in the mRNA expression of these receptors in MONO-MAC-6 cells with or without biofilm stimulation in this study. Both HHA and in particular, OHA increased ICAM1 mRNA expression, which suggested that HA fragments and high-MW HA had a higher affinity to ICAM-1 in MONO-MAC-6 cells. The role of ICAM-1 in HA signaling is underlined by an *in-vitro* study with LPS-stimulated human U937 macrophages [51]. The decreasing effect of high-MW HA on pro-inflammatory cytokines as IL-1β, IL-6 and TNF-α was mitigated when an anti-ICAM-1 antibody was applied prior to HA incubation (Yasuda, 2007). The HA binding to ICAM-1 down-regulated p65 NF-kB phosphorylation without affecting MAPK pathways (Yasuda, 2007).

Periodontal ligament fibroblasts (PDLF) are also a crucial and prominent cell type in periodontal homeostasis and regeneration due to their ability to produce multiple cytokines in response to bacterial insults, including the proinflammatory chemokine IL-8 (El-Awady et al., 2010). IL-8 is among the most abundant chemokines in periodontitis which functions as attracting PMNs to infectious area and affecting bone metabolism (Sahingur and Yeudall, 2015). High IL-8 levels were found *in vivo* in periodontitis patients (Chen et al., 2015) and *in vitro* in bacteria-stimulated periodontal fibroblasts (Makkar et al., 2022). In the present study, biofilm stimulation enormously increased the level of IL-8 in PDLF

as well. The PDLF cells respond differently to HA depending on its MW. In this study, OHA promoted the production of IL-8 in resting PDLF time-dependently. Nakatani et al. revealed that HA oligomers increased matrix metalloproteinase-1 in PDLF via p38MAPK signaling pathway (Nakatani et al., 2009). These and our findings suggest that OHA may promote periodontal tissue degradation under the pathologic conditions. High-MW HA (1300 kDa) was reported to downregulate IL-8 in *P. gingivalis*-stimulated gingival fibroblasts (Chen et al., 2019). This study found a downregulation by HHA (1000 kDa) with or without biofilm stimulation, although the effect was not statistically significant.

As with the MONO-MAC-6 cells, an influence of HA on its receptor expression was only found for ICAM-1. Most expression was stimulated by the periodontal biofilm. The increased expression of ICAM-1 in periodontal fibroblasts was shown for *P. gingivalis*, a member of our multi-species biofilm (Liu et al., 2014). In the presence of biofilm, not any HA could significantly influence the expression underlying the overwhelming role of bacteria. Our data indicated that in the absence of bacterial stimuli, OHA increased the expression of ICAM-1 and thus may support inflammation in the periodontium. In periodontal junctional epithelium, a gradient expression of ICAM-1 together with the high releasing of IL-8 are thought to be an important mechanism for guiding PMNs to infected areas, for example: the bottom of sulcus, where they are directly challenged by bacteria and their components (Tonetti et al., 1998).

ROS generation plays a critical role in numerous diseases, including periodontitis. ROS kills bacteria in high amounts, but when overactivated, it becomes cytotoxic to host cells, leading to tissue destruction (Sies et al., 2022). Several studies have shown a direct correlation between elevated oxidative stress and periodontitis (Sczepanik et al., 2020). Our data showed that periodontal biofilm induced oxidative stress in host immune cells as evidenced by the higher ROS levels in both MONO-MAC-6 and PDLF. Addition of antioxidants to conventional approaches may be an option for the prevention and treatment of periodontitis (Sczepanik et al., 2020). In the present study, HHA slightly retarded the high ROS generated by biofilm stimulation. High-MW HA was shown to have antioxidant capacity by scavenging excessive ROS (Šoltés et al., 2006). Clinically, adjunctive application of high-MW HA led to a higher increase in antioxidant markers in saliva when compared to non-surgical periodontal therapy without adjunct (Olszewska-Czyz et al., 2022).

It is worth to note that in the context of two immune cell types, the two high-MW HA affect differently on inflammatory response including cytokine expression and ROS generation. Without biofilm stimulation, CHA reduced IL-10 levels in MONO-MAC-6 cells and IL-8 levels in PDLF indicating a potential inhibitory effect of CHA on immune cells. In biofilm-stimulated MONO-MAC-6 cells, CHA reduced IL-1β levels less than HHA but increased IL-10 levels more. According to the manufacturer's information, CHA contains the crosslinker 1,4-butanediol diglycidyl ether (BDDE). BBDE is one of the most used cross-linker in commercial HA products (De Boulle et al., 2013). It might be responsible for the higher level of IL-1beta in CHA compared to HHA. BBDE has been shown to induce higher levels of pro-inflammatory cytokines, such as TNF-α and IL-1β than other crosslinkers in human keratinocyte cell line and human dermal fibroblast cell line (Jeong et al., 2021). Higher levels of ROS were observed in CHA group than in HHA group. The crosslinked structure appeared to be a feasible way to protect the long HHA chains from rapid degradation by free ROS (De Boulle et al., 2013).

In summary, within the tested 12-species periodontal biofilm, both high-MW HAs exhibited anti-biofilm capacity against early-stage and mature biofilms, with CHA demonstrating the most significant effect. Concerning periodontal immune cells and their interaction with biofilm, OHA might initiate a pro-inflammatory response in both MONO-MAC-6 cells and PDLF, whereas CHA inhibited it. When challenged with biofilm, HHA and CHA reduced pro-inflammatory IL-1β, while CHA increased the level of the anti-inflammatory IL-10. Expression of ICAM-1 is involved in the interaction between HA, biofilm, and MONO-MAC-6 as well as PDLF cells.

Nevertheless, this study has limitations. While this study is the first to demonstrate the anti-biofilm effects of HHA and CHA on the 12-species periodontal biofilm with defined strains, we acknowledge that we did not investigate the complexity as it occurs *in vivo*, nor the effect on the virulent factors of periodontal pathogens. Furthermore, the current study did not examine in detail how HA is involved in the M1/M2 switch of macrophages or in the potential signaling pathways. Despite the promising effect of HA on biofilm and immune cells, the effect of the biofilm and immune cells on HA was not examined. Future studies on this topic are of interest.

Overall, this study explored the effects of different types of HA, including variations in molecular weight (MW) and cross-link formulations, on periodontal biofilm, immune cells, and their interactions. The findings suggest that utilizing different types of HA at specific times and conditions during periodontal treatment may enhance the benefits of HA and improve clinical outcomes. However, further evidence is needed, and additional studies should focus on this aspect.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies involving humans because According to the criteria of the Cantonal Ethical Committee (KEK), there is no need for additional approval if the biomaterials are classified as "irreversibly anonymized". The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. AS: Resources, Supervision, Writing –

review & editing. SE: Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Histological evaluation of nonsurgical periodontal treatment with and without the use of sodium hypochlorite / amino acids and cross-linked hyaluronic acid gels in dogs

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Abstract

Objectives To evaluate periodontal wound healing following scaling and root planing (SRP) in conjunction with the application of sodium hypochlorite/amino acids and cross-linked hyaluronic acid (xHyA) gels in dogs.

Materials and Methods In four beagle dogs, 2-wall intrabony defects were created and metal strips were placed around the teeth. Clinical parameters were measured 4 weeks after plaque accumulation. The experimental root surfaces were subjected to SRP with either the subgingival application of a sodium hypochlorite/amino acid gel and a xHyA gel (test group) or SRP alone (control group) using a split-mouth design. Clinical parameters were re-evaluated at 6 weeks. The animals were sacrificed at 8 weeks for histological analysis.

Results The test group showed significant improvements in all clinical parameters compared to the control group. Histologically, the test group exhibited statistically significantly greater new bone formation [i.e., length of newly formed bone, new bone area] compared with the control group ($p < 0.05$). Furthermore, statistically significantly greater formation of new attachment [i.e., linear length of new cementum adjacently to newly formed bone with inserting collagen fibers] and new cementum was detected in the test group compared with the control group at 8 weeks ($p < 0.05$ and $p < 0.01$, respectively).

Conclusion The adjunctive subgingival application of sodium hypochlorite/amino acid and xHyA gels to SRP offers an innovative novel approach to enhance periodontal wound healing/regeneration.

Clinical relevance The present findings have for the first-time shown histologic evidence for periodontal regeneration in support of this novel treatment modality.

Keywords Periodontitis · Periodontal wound healing/regeneration · Non-surgical periodontal therapy · Cross-linked hyaluronic acid · Sodium hypochlorite/amino acids

Introduction

Periodontitis is a highly prevalent chronic inflammatory disease caused by dysbiotic dental plaque, leading to the destruction of connective tissue attachment and loss of alveolar bone, ultimately resulting in tooth loss [1–3]. The treatment of periodontitis is multi-staged and first steps of non-surgical periodontal therapy (NSPT) include supragingival plaque control, followed by subgingival scaling and root planing (SRP), aimed at eliminating biofilms, endotoxins, and calculus. This treatment reduces inflammation and reestablishes a favorable environment for oral hygiene measures [4–6]. The efficacy of SRP has been well reported by gains in clinical attachment level (CAL), reductions in periodontal pocket depth (PPD) and in the frequency of

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bleeding on probing (BOP) [4, 7, 8]. However, SRP does not always result in closure of periodontal pockets and the outcomes may be influenced by several patient related factors (e.g., smoking level, oral hygiene), anatomical factors (e.g., tooth type and surface, furcation involvement) and operator's experience [9–11]. The available evidence from histological studies indicates that SRP typically leads to a reparative type of healing characterized by formation of a long junctional epithelium and limited or no regeneration of cementum, periodontal ligament and bone [12–16]. Consequently, various strategies including the use of antibiotics, antiseptics and different biological agents adjunctive to SRP have been used to effectively control the bacterial biofilm caused inflammation and enhance wound healing [15, 17–25].

Recently, a novel formulation of sodium hypochlorite (NaOCL) gel buffered with leucine, lysine, and glutamine acid (Perisolv®, Regedent AG, Zürich, Switzerland) has been suggested as an adjunct to SRP. Since it has been shown that the active ingredients in the gel create chloramines, which have a strong antimicrobial effect and can penetrate the biofilm [26], it has been suggested that its use may aid for both the mechanical removal of hard and soft subgingival bacterial deposits and the detoxification of the root surface [26, 27]. In this respect, positive clinical effects of a sodium hypochlorite gel were reported in studies treating deep pockets at teeth [28, 29] and dental implants [27].

Hyaluronic acid (HyA) is a major natural glycosaminoglycan component of the extracellular matrix in many tissues such as skin, joints, eyes, and periodontium and has several unique physiochemical and biological properties including hygroscopic, viscoelastic, bacteriostatic, anti-inflammatory, anti-oedematous, pro-angiogenetic and osteoinductive nature [30–35]. HyA is currently also available in cross-linked form (cross-linked HyA: xHyA) for various applications in tissue engineering, serving as biologics/scaffolds to further improve the overall mechanical properties and provide a longer degradation period compared with non-cross-linked HyA [36, 37]. Results from clinical studies, indicate positive outcomes evidenced by significant gain of CAL, PPD reduction and improved BOP values have been reported following the adjunctive application of xHyA to nonsurgical and surgical periodontal therapy [17, 31, 38]. Furthermore, a recent series of preclinical studies has demonstrated periodontal regeneration, evidenced by formation of cementum, periodontal ligament, and bone, following the application of xHyA in conjunction with reconstructive periodontal surgery for recessions, intrabony, and furcation defects [39–41].

Very recently, a novel two-step approach consisting of enhanced biofilm removal during nonsurgical therapy by means of a sodium hypochlorite/amino acid followed by application of a xHyA gel was suggested to improve the outcomes of nonsurgical periodontal therapy [42–44]. Results from two case series have shown statistically significant clinical improvements compared to baseline following SRP in conjunction with sodium hypochlorite/amino acid and xHyA [42, 43], thus suggesting that this strategy may represent a valuable novel strategy in non-surgical periodontal treatment. Additionally, a randomized controlled clinical study has assessed the clinical outcomes achieved through either mechanical subgingival debridement in conjunction with a sodium hypochlorite/amino acids-containing gel followed by subsequent application of a xHyA gel, or mechanical debridement alone [44]. The results have shown that both treatments led to statistically significant improvements in all evaluated clinical parameters, but the adjunctive subgingival application of sodium hypochlorite/amino acids and xHyA to SRP resulted in statistically significantly greater improvements compared to SRP alone [44].

However, to the best of our knowledge, at present no histological data are available evaluating the healing following the use of this novel approach for non-surgical therapy. Therefore, the aim of this study was to histologically evaluate in dogs, the healing following nonsurgical periodontal therapy and in conjunction with sodium hypochlorite/amino acid gel and xHyA application.

Methods and materials

Animals

Four healthy male beagle dogs, 26 to 38 months of age and weighing 9 to 15 kg, were used in this study. The animals were housed and monitored daily for the duration of the study in the Animal Experimentation Facility Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan. They were kept in individual cages at 20–26 ℃, relative humidity of 30–70%, and a 12-h light/dark cycle. Approximately 300 g of solid food (NVE-10, Nippon Pet Food, Co., Ltd. Tokyo, Japan) was provided to each animal daily and water was available ad libitum. All procedures during the in-life phase were approved by the ethical committee of the Animal Research Center of Kagoshima University, Japan (Project Approval No. D22017; Date of approval: 23 January 2023). This study conformed to the ARRIVE guidelines for preclinical animal studies.

Induction of experimental periodontitis

All surgical procedures were performed under general and local anesthesia using aseptic routines by one experienced surgeon (Yo.S.). Before surgical procedures, antibiotics (dihydrostreptomycin sulfate aqueous suspension for injection, 0.05 ml/kg; Mycillin Sol Meiji for veterinary use, Meiji Seika Pharma Co. Ltd, Tokyo, Japan) were administered intramuscularly. General anesthesia was induced with

intramuscular injection using medetomidine hydrochloride (Domitor®, 0.08 ml/kg IM; Orion Corporation, Espoo, Finland), 0.08 ml/kg of midazolam (Dormicum®, IM; Maruichi Pharmaceutical, Osaka, Japan) and 0.02 ml/kg of butorphanol tartrate (Vetorphale® 5 mg, Meiji Seika Pharma, Tokyo, Japan). After sedation, the anesthesia was maintained by inhalation of sevoflurane (0.5%–5.0%, Mylan Pharma Co., Ltd. Osaka, Japan) and a nitrogen/oxygen (2:1) mixture using an intracircuit vaporizer for spontaneous breathing. Local anesthesia was achieved with lidocaine HCl/epinephrine (2%, 1:80,000; Xylocaine; Fujisawa Inc., Osaka, Japan). The bilateral mandibular first and third premolars were carefully extracted to provide enough space for creation of intrabony defects. After a 8-week healing interval, two-wall intrabony defects (5 mm wide and 5 mm deep) were prepared bilaterally at the mesial aspect of the mandibular fourth premolars (P4) and at the distal aspect of the mandibular second premolars (P2) (four defects per dog). Following elevation of the mucoperiosteal flap, defects were created by using fissure burs with a sterile saline coolant (Fig. 1a). Cementum was removed using Gracey curettes and a chisel. Reference notches were made with a #1 round bur on the root surface at the cementoenamel junction (CEJ), and on the crown surface, to indicate the precise center plane of the intrabony defects and to allow an optimal histomorphometric analysis. To prevent spontaneous healing and induce plaque accumulation, metal strips were fixed to the tooth surface in the intrabony defects with a self-cure dental adhesive resin cement (Super Bond C&B, Sun Medical Co., Ltd., Moriyama, Japan) (Fig. 1b). The flaps were repositioned and stabilized with 4–0 silk sutures. Ketoprofen for analgesia (Capisten IM 50 mg, 2 mg/kg, 0.1 ml/kg; Kissei Pharmaceutical Co. Ltd, Matsumoto, Japan) and an antibiotic (Mycillin Sol) were administered daily for 2 days following the surgeries. Intraoral periapical radiographs at selected sites including the teeth (P2 & P4) were taken immediately after the treatment. The sutures were removed after 14 days of healing. To promote plaque formation, the animals were fed a soft diet during the induction period (Fig. 1c). After 4 weeks, bone loss progression was confirmed by the radiographs and the metal strips were removed without flap reflection. Acrylic stents with a groove on the mid-proximal root surfaces where the deepest pockets were detected were then fabricated to standardize the location of periodontal probe for clinical measurements (Fig. 1d) during this study.

Non-surgical periodontal therapy

Plaque control was performed with routine (3 times a week) flushing of the oral cavity with 2% chlorhexidine gluconate solution for 2 weeks prior to the treatment. To prevent mixing of gel type agents to the other site in the same side of the mandible, split-mouth design was employed in this study. Experimental 16 teeth (i.e., bilateral P2 & P4) were designated test and control side by coin flipping. Immediately before SRP, professional supragingival mechanical tooth cleaning was performed for the teeth. On one side, teeth were treated by SRP with sodium hypochlorite/amino acid gel followed by a cross-linked hyaluronic acid (xHyA) gel application (test group), whereas teeth of the contralateral side were treated by SRP only (control group) by the same experienced operator (T.N.). In the test group, SRP was performed as follows: in the teeth a sodium hypochlorite/amino acid gel (Perisolv®, an alkaline 0.95% sodium hypochlorite solution and a slightly viscous alkaline gel containing glutamic acid, leucine, lysine, carboxymethyl cellulose and titanium dioxide, Regedent AG, Zürich, Switzerland) was instilled into the periodontal pockets using a blunt needle for

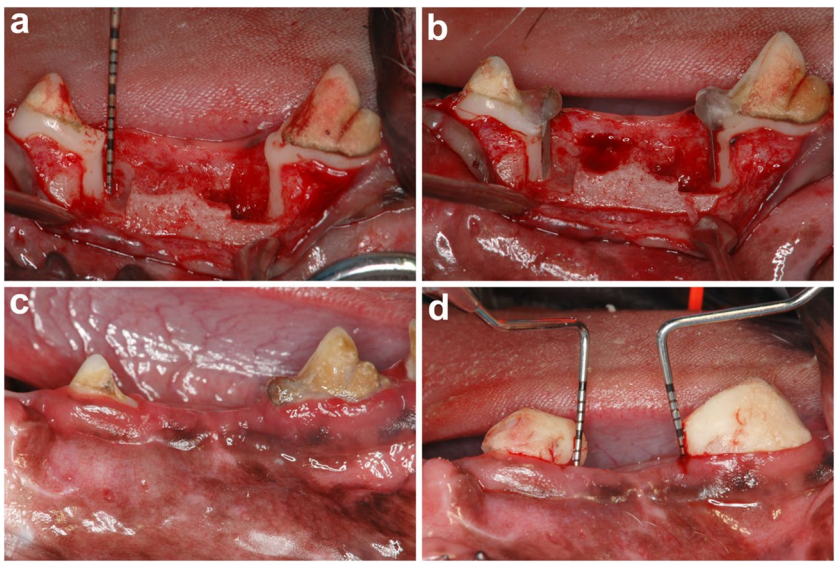

Fig. 1 **a** Surgically created two-wall intrabony defects in mandible. **b** Placement of the metal strips on the denuded root surfaces. **c** Plaque accumulation after 4 weeks. **d** Standardized measurement of clinical parameters by using customized acrylic stents with guiding grooves at baseline

30 s (Fig. 2a) prior to saline irrigation and instrumentation using an ultrasonic device (ENAC 10WA, Osada, Tokyo, Japan) and an ultrasonic tip (ST35, Osada, Tokyo, Japan) for 15 s (Fig. 2b), followed by hand instrumentation with manual curettes (LM Sharp Diamond Mini Gracey 11/12, 13/14 SD curettes, LM Dental™, Finland) through 5 traction movements in buccal and interproximal area (Fig. 2c), and the same process was repeated again. Following the final saline irrigation, the xHyA (hyadent BG®, a gel formulation containing butanediol diglycidyl ether-cross-linked HA (1000 kDA HA monomers) and non-cross-linked HA (2500 kDA) in a ratio 8:1, made from biotechnologically produced synthetic HA, REGEDENT AG, Zurich, Switzerland) gel (0.1 ml/tooth) was instilled in the pockets using a blunt needle (Fig. 2d). Teeth in the control group underwent the identical procedure except the sodium hypochlorite/amino acid and xHyA application. After the treatments, no antibiotics or analgesics were administrated, and the animals were fed a hard diet and the aforementioned oral hygiene regimen was performed daily for 8 weeks to reduce plaque formation.

Clinical evaluation

The following clinical parameters were assessed using a periodontal probe (UNC 15 Hu-Friedy, Chicago, IL., USA) to the nearest mm on all teeth at one site per tooth by one experienced and blinded examiner (F.S.) at baseline (Fig. 1d) and 6 weeks following the treatment: (a) probing pocket depth (PPD), (b) clinical attachment level (CAL) measured from the acrylic stent margin to the bottom of the probed pocket and (c) bleeding on probing (BOP). BOP was evaluated simultaneously with PPD by recording the presence (+) or absence (-) of bleeding up to 15 s after probing.

Histologic preparation

Eight weeks after the non-surgical therapy, intraoral radiographs were taken, and the animals were euthanized with an overdose of sodium thiopental. The teeth were removed together with the surrounding soft and hard tissues. The tissue blocks were fixed in 10% buffered formalin, trimmed according to intraoral radiographs and the reference notch on the crown, and rinsed in phosphate-buffered saline. The samples were decalcified in Kalkitox™ (Wako Pure Chemical Industries, Ltd., Osaka, Japan), dehydrated, and embedded in paraffin. Serial 6-µm-thick sections were then prepared along the mesiodistal plane and were stained with hematoxylin and eosin or with azan.

Histomorphometric analysis

All specimens were analyzed under a light microscope (BX51; Olympus Corp., Tokyo, Japan) equipped with a computerized image system (WinROOF2015; Mitani Corporation, Tokyo, Japan). For histomorphometric analysis, three sections approximately 90 µm apart were selected from the most central area of each two-wall defect, identified by the length of the root canal and the reference notches. The mean value of each histomorphometric parameter was then calculated for each site. To evaluate intra-examiner reproducibility, sixteen sections from all sites were read by a single blinded examiner at two different moments (48 h apart), and inter-calibration of the examiner was accepted at 90% level. The following parameters were measured by the examiner (T.I.). 1. Defect height (DH): distance between the apical extent of root planing and the CEJ. 2. Junctional epithelium length (JE): distance between the apical extension of the junctional epithelium and the CEJ. 3. Connective

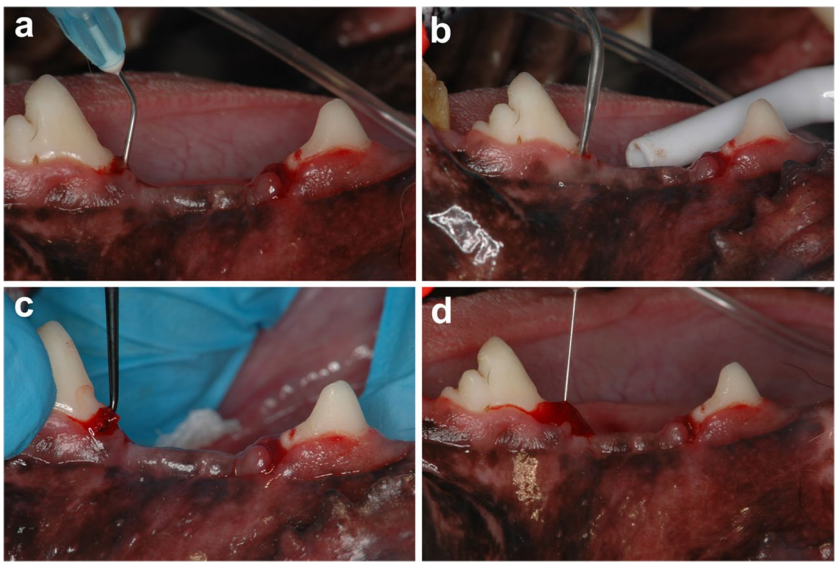

Fig. 2 **a** Application of sodium hypochlorite/amino acid gel to the periodontal pocket. SRP performed using an ultrasonic device with an ultrasonic tip (**b**) and hand instruments (**c**). **d** Application of a xHyA gel to the periodontal pocket

tissue adhesion (without cementum) (CT): distance between apical extent of the junctional epithelium and the coronal extent of the newly formed cementum. 4. New bone length (NB): distance between the apical extent of root planing and the coronal extent of newly formed alveolar bone along the root surface. 5. New bone area (NBA): newly formed trabecular bone within a template (5 × 5 mm) that served as a standardized proxy for the defect site. The template was aligned parallel to the root surface interfacing the apical extension of the root planing [45]. 6. New cementum length (NC): distance between apical extent of root planing and coronal extent of newly formed cementum on the denuded root surface.

7. New attachment length (NA): linear length of the root surface covered by NC adjacent to newly formed bone, with functionally oriented collagen fibers.

8. Periodontal ligament score (PDL score): which was obtained by grading the periodontal ligament with the reported scoring system outlined by Wikesjö et al. [46].

Statistical analysis

The primary outcome of this study was the histomorphometric outcome in terms of NA, measured for the treatment groups at 8 weeks. Clinical parameters were evaluated as secondary outcomes. However, due to the limited number of pre-clinical studies in dogs with a comparative design and primary outcome, no power analysis for sample size calculation could be performed. For obvious ethical reasons, sample size was set to an absolute minimum (4 animals) and the animal was chosen as the unit for the statistical analysis. The means and standard deviations for each parameter were calculated for each of the treatment groups. Wilcoxon signed rank test was used to compare the clinical parameters between baseline and at the 6 week follow up. Mann–Whitney U test was used to compare the clinical and histological values between the control and test groups.

For the comparison of the proportions of sites showing bleeding on probing (BOP), Fisher's exact test was used. A P value of < 0.05 was considered statistically significant. All calculations were performed with statistical software (BellCurve for Excel; Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Clinical observations

Postoperative clinical healing was uneventful at all 16 (8 sites/group) sites in the control and test groups. No visible adverse reactions, including suppuration, abscess formation, or increased tooth mobility, were observed throughout the entire experimental period. Visual gingival redness seemed to remain longer or recur in the control group after the treatment (Fig. 3).

Clinical measurements

The values for clinical parameters at the baseline and 6-week examinations in both treatment groups are shown in Table 1. The baseline examination revealed that the two study groups demonstrated similar characteristics for PPD, CAL, and BOP score with no significant differences between the groups. Both treatment groups showed clinical improvements at 6 weeks compared to baseline. Mean PPD reduction between the baseline and 6 weeks follow-up was statistically significantly different between the groups in favor for the test group ($p < 0.05$). The test group showed better results in terms of mean CAL gain compared to control group, however, no statistically significant difference was detected between the groups. The number of bleeding (BOP +) sites was markedly reduced in the test group at 6 weeks compared to baseline. Similarly, there was statistically significant difference in the score between the groups.

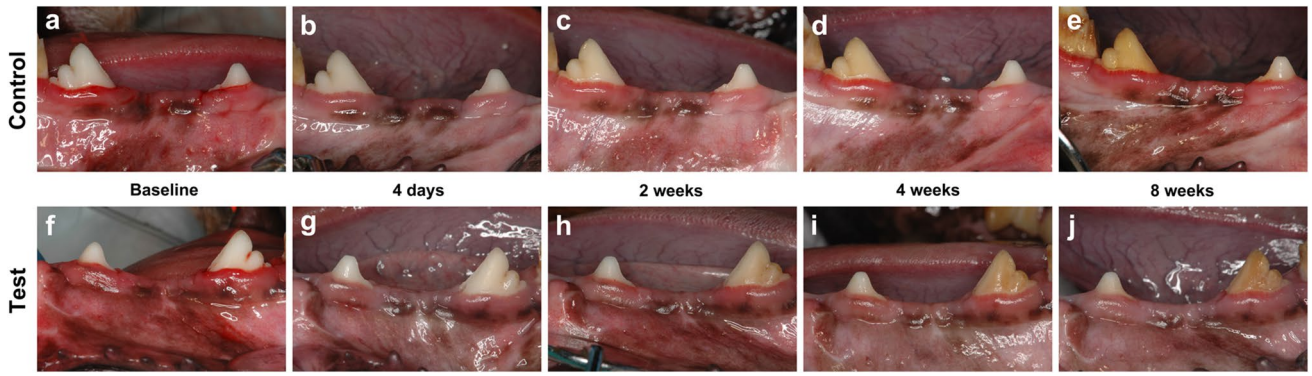

Fig. 3 Clinical overview at baseline, 4 days, 2, 4 and 8 weeks in the control (**a**-**e**) and the test group (**f**-**j**)

Table 1 Clinical parameters for each treatment at baseline and 6 weeks (means ± SD)

Parameters	Control	*N* = 4 animals Test
PPD (mm)		
Baseline	5.46 ± 0.82	5.50 ± 0.57
6 weeks	2.12 ± 0.32	1.25 ± 0.50
PPDreduction	3.34 ± 0.54	4.25 ± 0.50[†]
CAL (mm)		
Baseline	5.71 ± 0.84	5.59 ± 0.47
6 weeks	2.93 ± 0.23	1.68 ± 0.89
CAL gain	2.78 ± 0.79	3.90 ± 0.82
BOP (+) n (%)		
Baseline	8 (100)	8 (100)
6 weeks	6 (75.0)	1 (12.5)[*] [†]

PPD probing pocket depth, *CAL* clinical attachment level, *BOP* bleeding on probing

[*] Significantly different from baseline within each group ($p<0.01$)

[†] Significantly different from control group ($p<0.05$)

Descriptive histology

In the control group, a collapse of the soft tissue could be observed in three teeth and the oral gingival epithelium was partially thickened with deeper rete ridges than normal one (Fig. 4a and b) in four teeth. Also, most sites (seven out of eight teeth) showed the slight to moderate widespread inflammatory cell infiltrate mostly at the tips of gingiva (Fig. 4a and b). Downgrowth of junctional epithelium was detected slightly below the CEJ (Fig. 4a and e). Periodontal defect was mostly occupied by fibrous connective tissue (Fig. 4a and c) and slight superficial (two teeth) and inflammatory (one tooth) root resorption areas were seen on the root surfaces without cementum formation (Fig. 4e and f) in three teeth. Extensive proximal host bone resorption and varying degrees of spontaneous bone formation occurred along the root surfaces (Fig. 4a and d) in all sites. Two teeth in the control group presented no cementum formation at all and predominantly acellular cementum formation was restricted at the apical extension of instrumentation (Fig. 4a, f and g) in five teeth. Most specimens (5/8, 62.5%) in the control group showed non-functional disordered periodontal ligament like tissue or collagen fibers detached from the root surfaces (Figs. 4f and g and Fig. 6a).

In the test group, residual xHyA with reticular appearance was well integrated with gingival connective tissue at the coronal portion of the defects in all sites (Fig. 5a and b). Some remnants of xHyA were observed around/in the newly formed bone and occasionally between new cementum and new bone (Fig. 5a, c, d and f). Marked soft tissue atrophy or

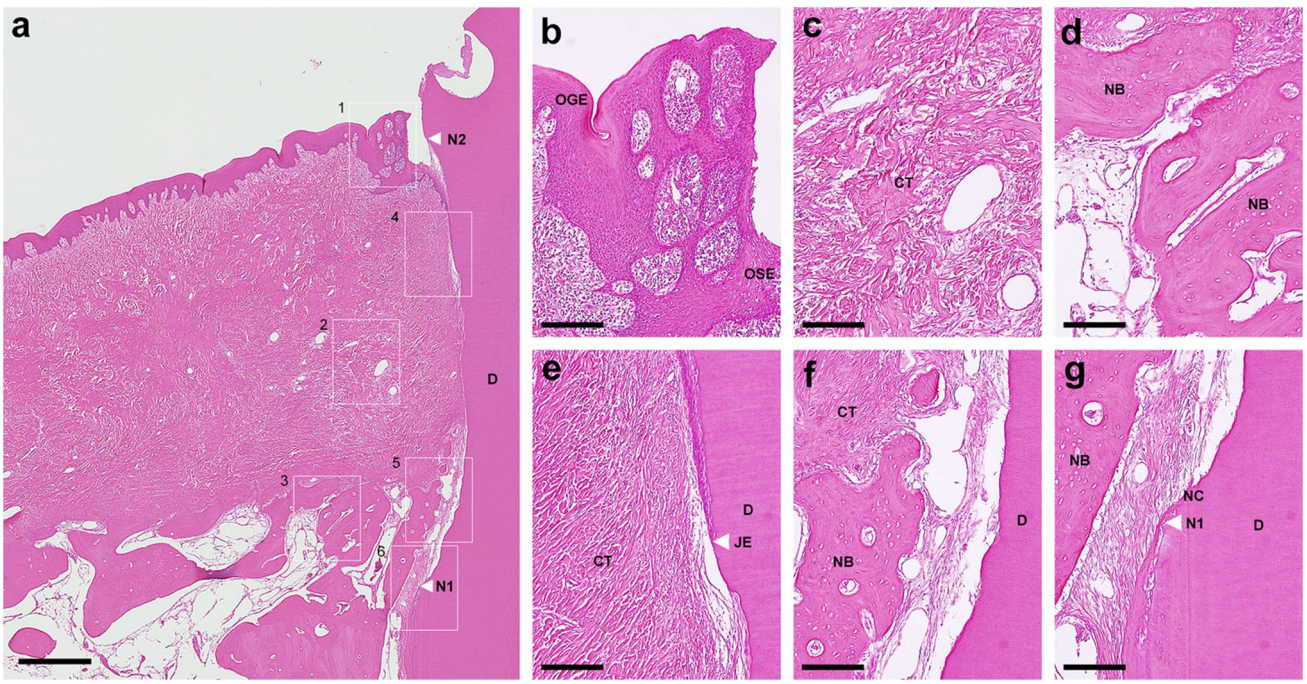

Fig. 4 **a** Histologic overview of defect treated with SRP alone (control group). (scale bar, 1 mm; hematoxylin and eosin stain). **b** Higher magnification of the box 1 area. **c** Higher magnification of the box 2 area. **d** Higher magnification of the box 3 area. **e** Higher magnification of the box 4 area. **f** Higher magnification of the box 5 area. **g** Higher magnification of the box 6 area. (scale bar, 200 μm; hematoxylin and eosin stain). D, root dentin; N_1, apical end of root planing; N_2, cementoenamel junction; OGE, oral gingival epithelium; OSE, oral sulcular epithelium; JE, apical end of junctional epithelium; CT, gingival connective tissue; NB, new bone; NC, new cementum

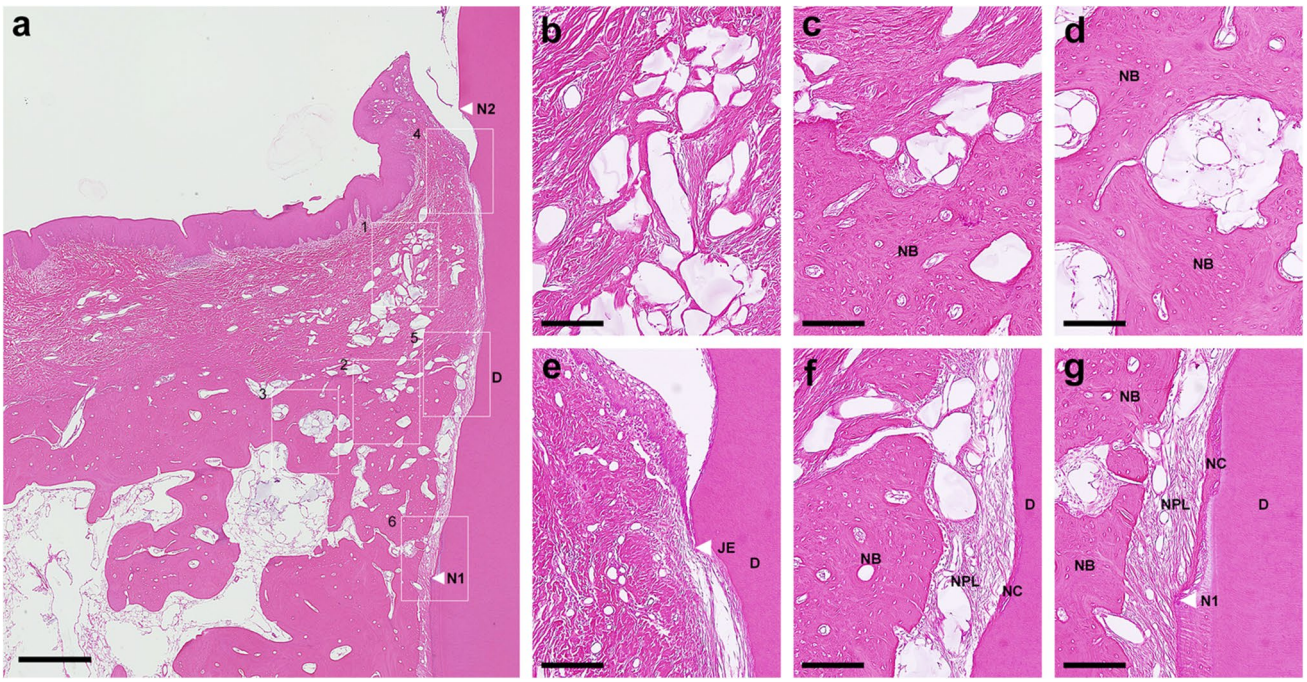

Fig. 5 **a** Histologic overview of defect treated with SRP with a sodium hypochlorite and amino acids gel and a cross-linked hyaluronic acid gel (xHyA) gel (test group). (scale bar, 1 mm; hematoxylin and eosin stain). **b** Higher magnification of the box 1 area. **c** Higher magnification of the box 2 area. **d** Higher magnification of the box 3 area. **e** Higher magnification of the box 4 area. **f** Higher magnification of the box 5 area. **g** Higher magnification of the box 6 area. (scale bar, 200 µm; hematoxylin and eosin stain). D, root dentin; $N_{1,}$ apical end of root planing; $N_{2,}$ cementoenamel junction; JE, apical end of junctional epithelium; CT, gingival connective tissue; NB, new bone; NC, new cementum; NPL, new periodontal ligament

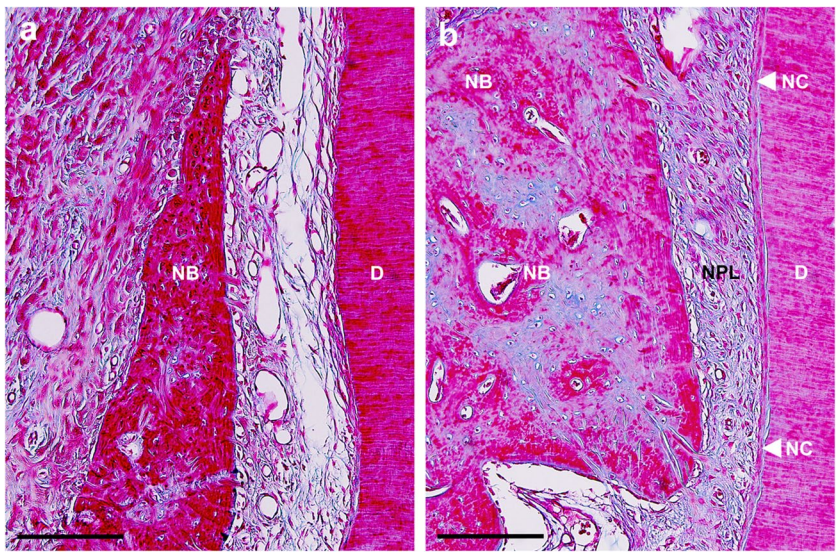

Fig. 6 **a** Higher magnification view of the middle portion of the defect treated with SRP alone (control group). Loosely arranged collagen fibers were seen near the root dentin without new cementum. (scale bar, 200 µm; azan stain). **b** Higher magnification view of the middle portion of the defect treated with SRP with a sodium hypochlorite and amino acids gel and a cross-linked hyaluronic acid gel (xHyA) gel (test group). Dense obliquely oriented collagen fibers were observed between the new bone and cementum. (scale bar, 200 µm; azan stain). D, root dentin; NB, new bone; NC, new cementum; NPL, new periodontal ligament

pathological change of gingival epithelium was not noted (Fig. 5a). A limited inflammatory response was observed in the tips of gingiva around three teeth (Fig. 5e). Apical extension of junctional epithelium was mostly restrained at the CEJ (Fig. 5a and e). Superficial root resorption was detected on the root surface of one tooth. New bone formation extended from the host bone toward the coronal region of the defects (6/8, 75%) (Fig. 5a). Newly formed bone was well integrated with the original bone and characterized by cancellous bone, which consists of a network of bony trabeculae containing bone marrow, blood vessels, osteoblasts, and osteocytes (Fig. 5a, c and d). A continuous layer of new cellular/acellular cementum was seen, with or without inserting collagen fibers running perpendicular to the root surfaces, was observed covering half of the defect area (Fig. 5a, f and g) in 5 teeth. The highly vascularized and dense new periodontal ligament-like tissue, which was formed between the new cementum and new bone (Figs. 5f and gandFig. 6b), maintained its width up to the coronal portion in the test group. No ankylosis was observed in any of the teeth.

Histomorphometric analysis

The results of histomorphometric analysis are shown in Table 2. No statistically significant differences were detected between the groups in regard to the following measurements (DH, JE, and CT). However, the length of CT (without cementum formation) in the test group was smaller than that observed in the control group. The length of new cementum was statistically significantly ($P < 0.01$) greater in the test (2.46 ± 0.77 mm) group than in the control (0.85 ± 0.84 mm) group. The test (1.75 ± 0.65 mm) group yielded statistically significantly ($P < 0.05$) greater formation of new attachment (i.e., linear length of NC adjacent to newly formed bone, with functionally oriented collagen fibers) compared with control (0.48 ± 0.79 mm) group. Moreover, the PDL scores in the test (2.87 ± 1.59) group was statistically significantly ($P < 0.05$) higher than that in the control (1.00 ± 0.94) group. The amount of newly formed bone (e.g., the length of NB and the area of NB) in the test (3.01 ± 0.64 mm and 5.75 ± 2.21 mm^2, respectively) group was statistically significantly ($P < 0.05$) greater than that in the control (2.26 ± 0.64 mm and 3.14 ± 1.94 mm^2, respectively) group.

Table 2 Histomorphometric comparisons between test and control groups 8 weeks after treatment. (means ± SD)

Parameters	Control	$N = 4$ animals Test
DH (mm)	5.60 ± 0.42	5.77 ± 0.52
JE (mm)	1.22 ± 0.45	1.03 ± 0.31
CT (mm)	2.84 ± 1.33	1.86 ± 1.00
NC (mm)	0.85 ± 0.84	2.46 ± 0.77**
NA (mm)	0.48 ± 0.79	1.75 ± 0.65*
PDL score (1–5)	1.00 ± 0.94	2.87 ± 1.59*
NB (mm)	2.26 ± 0.64	3.01 ± 0.64*
NBA (mm^2)	3.14 ± 1.94	5.75 ± 2.21*

DH defect height, *JE* junctional epithelium length, *CT* connective tissue adhesion (without cementum), *NB* new bone length, *NBA* new bone area, *NC* new cementum length, *NA* new attachment length, *PDL score* periodontal ligament score

* Significantly different from control group ($p < 0.05$)

** Significantly different from control group ($p < 0.01$)

Discussion

The present study has, for the first time, provided histological evidence of periodontal regeneration following the adjunctive subgingival application of sodium hypochlorite/amino acids and a xHyA gels to SRP. The histological results were consistent with the greater clinical improvements observed in terms of PPD reduction, CAL gain, and reduction of inflammation in the test group compared to the control group, which in tern, underscores the potential clinical significance of these findings.

The clinical findings obtained in this animal study are in line with the results from clinical studies reporting that SRP combined with sodium hypochlorite/amino acid and xHyA gels resulted in statistically significantly higher clinical improvements evidenced through PPD reduction, CAL gain, and decrease of BOP score (values) as compared to baseline [42, 43] or SRP alone [44]. In line with the clinical findings, the histologic analysis revealed that the test treatment yielded statistically significantly greater amounts of new connective attachment and new cementum formation than the control one. In the defects treated by SRP with a sodium hypochlorite and amino acids containing gel and a xHyA gel, dense functionally oriented collagen fibers with numerous blood vessels were predominantly observed between the newly formed cementum and the newly formed bone showing high PDL scores compared to the teeth treated by SRP alone. In addition, statistically significantly greater new bone was measured in the test group compared with the control one.

When interpreting these positive results, it must be emphasized that the present study has used the combination of the two different materials as a single treatment adjunctive to SRP. The cleaning effect was expected by the sodium hypochlorite and amino acids gel, which create chloramines. Chloramines have a strong antimicrobial effect and minimize the effects of hypochlorite on sound dentin/root cementum and healthy soft tissue [26, 28, 47]. Additionally, in vitro, and clinical studies have demonstrated that the sodium hypochlorite/amino acid gel can facilitate SRP to disrupt the biofilm, by dissolving necrotic tissue, and by softening calculus and thus reducing

friction during instrumentation [26–29]. The positive effect on the healing was expected by subsequent application of a xHyA gel since several in vitro studies have demonstrated that HyA significantly stimulates blood clot formation [30, 48], induces angiogenesis [30, 33] and increases osteogenesis [30, 34] as a biological modulator for promoting periodontal wound healing/regeneration. Additionally, recent studies have shown that the surgical application of the same high molecular xHyA yielded statistically significant improvements characterized by PPD reduction and CAL gain in human intrabony defects [38] and effectively promoted periodontal tissue regeneration in canine 2-wall intrabony, gingival recession and class III furcation defects [39–41].

When interpreting the results it is important to point out that the study did not include treatment groups treated with sodium hypochlorite/amino acids gel or xHyA. Therefore, it is unclear to what extent each of the used adjunctive materials may have contributed to the favorable outcomes obtained in the test group. A very recent animal study has shown that treatment of experimental periodontitis in rats using the combination of sodium hypochlorite/amino acids gel and SRP yielded to better outcomes in terms of gingival bleeding index, tooth mobility and the overall aspect of the gingival structures than treatment with SRP alone. However, the histologic evaluation demonstrated a predominantly reparative type of healing characterized by non-functionally oriented collagen fibers and unrestored alveolar ridges following the treatment with sodium hypochlorite/amino acids gel and SRP [49]. Megally et al. reported that subgingival ultrasonic debridement with sodium hypochlorite/amino acid gel resulted in a clinically relevant PPD reduction and CAL gain in residual pockets in subjects in maintenance care. However, these improvements were comparable to that obtained in the ultrasonic debridement alone without statistically significant differences [28]. Also, Pilloni et al. demonstrated that subgingival instrumentation with the local adjunctive use of xHyA yielded statistically significant clinical and microbiological improvements compared to baseline in residual periodontal pockets, although there was a lack of statistically significant differences in the outcomes following the subgingival instrumentation with placebo control [50]. These results may justify the rationale for the novel approach including the combined use of the two materials adjunctive to SRP as a single treatment procedure. Additionally, it is important to emphasize that the present animal study was designed to assess the biologic potential of the very recently introduced clinical protocol for nonsurgical periodontal treatment [42–44].

An interesting observation in this study is related to the changes in bleeding scores; i.e., while gingival inflammation decreased within a week in both control and test groups, gingival swelling and redness gradually increased during the 8 weeks following treatment in the control group. On the contrary, no such increase occurred in the test group. This clinical observation was consistent with the histologically observed inflammatory cell infiltrate at the coronal part of gingiva in the control group. In the test group, varying degrees of xHyA remnants were consistently observed, but they did not appear to interfere with tissue integration in all periodontal defects around teeth. These findings are also in agreement with those from previous preclinical studies that have demonstrated the presence of residual xHyA in two-wall intabony and class III furcation defects in dogs [40, 41].

An important finding that warrants further attention is the fact that this novel approach for non-surgical periodontal therapy (NSPT) resulted in chronic two-wall intrabony defects in comparable amounts of NC (2.46 ± 0.77 mm) and NA (1.75 ± 0.65 mm) to those (3.20 ± 1.29 mm, 2.43 ± 1.29 mm respectively) obtained in the same initial size of acute two-wall intrabony defects treated with a surgical approach and the application of xHyA in dogs [40]. These findings indicate that the application of xHyA in NSPT is clinically beneficial, and the high molecular weight xHyA can maintain its stability for 4 to 8 weeks [40, 41, 51]. From a clinical perspective, the results of the present study suggest that this novel approach offer additional benefits in NSPT and decrease the need for surgical periodontal therapy.

In contemporary periodontology, the adjunctive use of EMD [20, 21], antibiotics [22, 23], lasers and antimicrobial photodynamic therapy [24, 25], has been repeatedly investigated as adjunctive approaches to NSPT. However, additional benefits compared to SRP alone were not consistently observed while the relatively high cost, some risks of allergy and the necessity of specific equipment for these treatment modalities need also to be considered. On the other hand, the novel approach evaluated in the present study is based on the use of a highly biocompatible and non-animal origin materials adjunctive to SRP. The sodium hypochlorite cleaning gel may offer further advantages to NSPT by facilitating the mechanical removal of the biofilm, thus enhancing the effects of the xHyA gel [42]. Moreover, the xHyA gel enhances blood clot stability and attracts several growth factors [38, 52, 53] which play a key role in periodontal wound healing/regeneration [54]. However, it needs to be emphasized that despite the fact that these results are encouraging, they were obtained in experimentally created defects including a small number of animals. Therefore, further studies are required to confirm the clinical relevance and the predictability of this novel treatment approach in NSPT.

Conclusion

In conclusion, the present data offer histological evidence supporting the use of adjunctive subgingival application of sodium hypochlorite/amino acid and xHyA gels in NSPT to enhance periodontal wound healing and regeneration.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and all procedures performed in studies involving animals were in accordance with the ethical standards of the ethical committee of the Animal Research Center of Kagoshima University, Japan (Project Approval No. D22017; Date of approval: 23 January 2023). This study conformed to the ARRIVE guidelines for preclinical animal studies. For this type of study, informed consent is not required.

Competing interests The authors declare no competing interests.

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Microbiological Effects of Sodium Hypochlorite/-Amino Acids and Cross-linked Hyaluronic Acid Adjunctive to Non-surgical Periodontal Treatment

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Purpose: To investigate the microbiological outcomes obtained with either subgingival debridement (SD) in conjunction with a gel containing sodium hypochlorite and amino acids followed by subsequent application of a cross-linked hyaluronic acid gel (xHyA) gel, or with SD alone.

Materials and Methods: Forty-eight patients diagnosed with stages II-III (grades A/B) generalised periodontitis were randomly treated with either SD (control) or SD plus adjunctive sodium hypochlorite/amino acids and xHyA gel (test). Subgingival plaque samples were collected from the deepest site per quadrant in each patient at baseline and after 3 and 6 months. Pooled sample analysis was performed using a multiplex polymerase chain reaction (PCR)-based method for the identification of detection frequencies and changes in numbers of the following bacteria: *Aggregatibacter actinomycetemcomitans* (A.a), *Porphyromonas gingivalis* (P.g), *Tannerella forsythia* (T.f), *Treponema denticola* (T.d), and *Prevotella intermedia* (P.i).

Results: In terms of detection frequency, in the test group, statistically significant reductions were found for P.g, T.f, T.d and P.i ($p < 0.05$) after 6 months. In the control group, the detection frequencies of all investigated bacterial species at 6 months were comparable to the baseline values ($p > 0.05$). The comparison of the test and control groups revealed statistically significant differences in detection frequency for P.g ($p = 0.034$), T.d ($p < 0.01$) and P.i ($p = 0.02$) after 6 months, favouring the test group. Regarding reduction in detection frequency scores, at 6 months, statistically significant differences in favour of the test group were observed for all investigated bacterial species: A.a ($p = 0.028$), P.g ($p = 0.028$), T.f ($p = 0.004$), T.d ($p < 0.001$), and P.i ($p = 0.003$).

Conclusions: The present microbiological results, which are related to short-term outcomes up to 6 months post-treatment, support the adjunctive subgingival application of sodium hypochlorite/amino acids and xHyA to subgingival debridement in the treatment of periodontitis.

Keywords: cross-linked hyaluronic acid, microbiology, non-surgical periodontal therapy, periodontitis, periopathogenic bacteria, sodium hypochlorite/amino acids

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Periodontitis is a chronic, inflammatory disease characterised by microbial dysbiosis, resulting in the destruction of connective tissue attachment and alveolar bone.[1,5,6,16] Periodontal treatment aims to reduce or eliminate the periodontal-pathogenic biofilm from the periodontal pockets and the surrounding periodontal tissues.[21] Therefore, the thorough

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mechanical disruption and removal of subgingival biofilm and calculus are key components of cause-related periodontal therapy, aiming to reestablish clinical health as evidenced by shallow probing depths and the absence of bleeding on probing.[34]

However, the complete removal of plaque and calculus is often limited due to anatomical factors (e.g., furcation involvement, deep pockets, anatomical grooves, or concavities), the operator's manual skills, and various patient-related factors (e.g., smoking status or systemic diseases). It has been demonstrated that up to 30% of the total surface area of subgingivally debrided roots may still be covered with residual plaque and calculus.[21] In order to further enhance the elimination of subgingival bacterial biofilm, various adjunctive materials with antimicrobial activity have been utilised.[27]

Recently, a novel concept termed "Clean and Seal" in conjunction with subgingival instrumentation has been suggested to improve the outcomes of non-surgical periodontal therapy.[10,25,26] The two constituents of "Clean and Seal" are sodium hypochlorite/amino acids (Perisolv, Regedent; Zürich, Switzerland) and cross-linked hyaluronic acid (high molecular) (xHyA) gels (Hyadent BG, Regedent).

Preclinical studies have shown that sodium hypochlorite is able to alter biofilm matrices and act in particular against Gram-negative species associated to periodontitis.[17] Moreover, favourable cell survival and spreading of periodontal ligament cells has been observed after the application of sodium hypochlorite/amino acids gel to root surfaces.[29] Clinically, the additive value of sodium hypochlorite/amino acids gel has been reported in treating deep periodontal pockets in untreated periodontitis,[15] residual periodontal pockets,[18,24] peri-implant-mucositis[14] and peri-implantitis.[28]

Preclinical evidence on cross-linked hyaluronic acid has demonstrated that this formulation is not only biocompatible with periodontal tissues but also enhances the proliferative, migratory, and wound healing properties of cells involved in soft-tissue wound healing.[3] Furthermore, cross-linked hyaluronic acid strongly induces the growth of osteoprogenitors and is able to maintain their stemness, thus potentially regulating the balance between self-renewal and differentiation during bone regeneration.[2] Importantly, histological evidence from animal studies revealed that the adjunctive application of cross-linked hyaluronic acid resulted in significant regeneration of periodontal tissues in treating intrabony defects, gingival recessions, or furcation defects as compared to surgical controls.[30-32] Findings from a systematic review have shown that the adjunctive application of hyaluronic acid to non-surgical periodontal treatment resulted in statistically significant improvements in probing depth reduction and gain in clinical attachment compared to controls.[11]

Recent findings from clinical studies have provided evidence indicating that the adjunctive application of sodium hypochlorite/amino acid and cross-linked hyaluronic acid gels to SD may result in significant clinical improvements, as evidenced by the reduction of probing pocket depths (PD), bleeding on probing (BOP), and gain in clinical attachment (CAL). This applies both to patients with untreated periodontitis and patients enrolled in maintenance but still exhibiting residual pockets.[10,25,26]

To the best of our knowledge, no clinical studies to date have reported on the microbiological outcomes following the treatment using this novel concept for non-surgical periodontal therapy. Therefore, the aim of this study was to investigate the potential microbiological advantages of this strategy in the treatment of periodontitis.

MATERIALS AND METHODS

Experimental Design

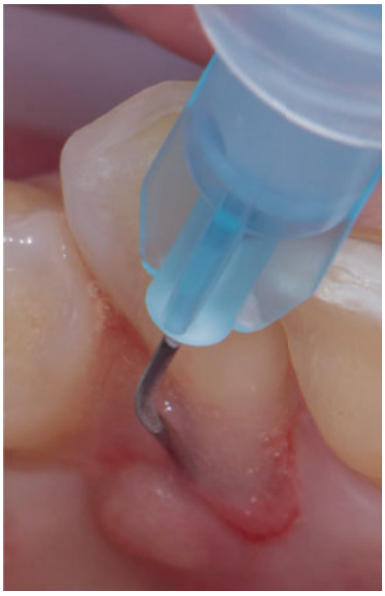
This randomised, controlled, parallel study included 48 non-smoking patients, diagnosed with stages II-III (grades A, B) generalised periodontitis, aged between 30 to 72 years (mean ± SD), who attended the Department of Dental and Oral Pathology at the Lithuanian University of Health Sciences in Kaunas, Lithuania, for periodontal treatment. The study's inclusion criteria were the absence of systemic diseases and no intake of medication which may affect periodontal health, the presence of at least 20 teeth, and absence of removable dentures. The study's exclusion criteria were: smokers, periodontal treatment during last 12 months, antibiotic treatment 3 months prior to the start of the trial, antibiotic prophylaxis required for dental treatment, pregnant/lactating women, and known allergies to sodium hypochlorite. The study protocol was registered at ClinicalTrials.gov, NCT04662216. All patients were enrolled between September 2019 and January 2022. Each patient was given detailed information of the study protocol and was required to sign an informed consent form.

Treatment Procedures

After an initial screening visit for recruitment and supragingival cleaning, patients were assigned randomly to the control or test groups (control group: 24 patients; test group: 24 patients). Demographic details, randomisation, allocation concealment and study design are described in detail in a related paper reporting clinical outcomes.[26] In brief, subjects in the control group underwent full-mouth SD performed with ultrasonic (Satelec/Acteon suprasson newtron ultrasonic scaler, Acteon; Norwich, UK) and hand instruments (LM SharpDiamond 1/2, 7/8, 11/12, 13/14 SD mini Gracey and Gracey curettes, LM; Parainen, Finland). Subsequently, all teeth were polished using a low-abrasive paste (Lunos Super Soft, RDA < 5, Dürr Dental; Bietigheim-Bissingen, Germany). In the test group, full-mouth SD was performed as follows: in all pockets with PD ≥ 4 mm, a sodium hypochlorite/amino acid gel (Perisolv, Regedent) was inserted into the pockets and left there for 60 s before subgingival instrumentation (Fig 1). Subgingival instrumentation was carried out with the same ultrasonic and hand instruments, and the application of sodium hypochlorite/amino acid gel was repeated until instrumentation was considered sufficient (i.e., a total of 2–3 times). Following SD and polishing, a mixture of natural and cross-linked hyaluronic acid (high molecular) gel (Hyadent BG, Regedent) was inserted in the pockets using a blunt needle (Fig 2).

Outcomes

The primary outcome variable was the change in detection frequency of *Aggregatibacter actinomycetemcomitans* (A.a), *Por-*



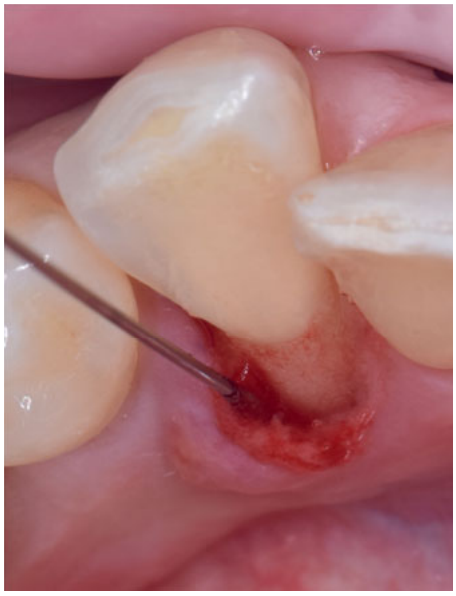


Fig 1 The application of sodium hypochlorite/amino acid gel to the periodontal pocket prior to subgingival debridement.

Fig 2 Application of a mixture of natural and cross-linked hyaluronic acid (high molecular) to the periodontal pocket after subgingival debridement.

phyromonas gingivalis (P.g), *Tannerella forsythia* (T.f), *Treponema denticola* (T.d), and *Prevotella intermedia* (P.i) from baseline to 6 months. Secondary outcome variables included the change of detection scores (0-4, which correspond to the number CFUs, see Table 1) of the respective bacteria as well as changes in PD, CAL, BOP and plaque index (PI) at sampled sites from baseline to 6 months.

Microbial Sampling

Subgingival plaque samples were collected at baseline (prior to SD) and at 3 and 6 months from the deepest pocket per quadrant by the same investigator (U.M.D). Following a thorough removal of supragingival plaque and calculus using periodontal curettes and sterile cotton pellets, each site was dried and isolated with cotton rolls. A sterile endodontic paper point ISO #30 (Dentsply Sirona; Bensheim, Germany) was inserted and left in place for 20 s. Four samples per patient were collected in a coded sterile-sealed Eppendorf tube and sent to the laboratory (Department of Laboratory Medicine, Lithuanian University of Health Sciences, Kaunas, Lithuania) for analysis. There, these samples were kept frozen at -20°C until further processing (for one day), and then at -80°C until the microbiological analysis was performed (not more than 30 days later). Molecular analysis of the subgingival plaque samples was performed manually in three steps:

- deoxyribonucleic acid (DNA) extraction;
- multiplex amplification with biotinylated primers;
- reverse hybridisation.

DNA Extraction

DNA extraction was performed using DNA purification from swab samples kit (Swab, version 0517, A&A Biotechnology; Gdynia, Poland). 700 µl of lysis solution and 20 µl of proteinase K were added to the original Eppendorf tubes containing the paper points with subgingival plaque samples. The tube contents were thoroughly mixed, briefly centrifuged, and incubated for 20 min at 37°C with mixing at 500 rpm. After incubation, the samples were mixed, centrifuged, and the resulting liquid was applied to the spin columns. The columns were centrifuged for 1 min at 12,000 rpm. Two washing cycles were performed using new 2-ml tubes and 500 µl of washing solution each time. The washing solution was centrifuged at 12,000 rpm for 1 min the first time and for 2 min the second time. The washed and spun columns were transferred to new 1.5-ml tubes, and 150 µl of elution buffer heated to 75°C was added, incubated for 3 min at room temperature, and centrifuged for 1 min at 12,000 rpm. The resulting DNA samples were stored at -80°C until further analysis.

Multiplex DNA Amplification

DNA samples were analysed using molecular genetic assay for combined identification of five periodontopathogenic bacterial species (micro-IDent VER 2.0, Hain Lifescience; Nehren, Germany) including *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia,* and *Treponema denticola*. Master mix of the amplification enzymes was freshly prepared before testing each batch of the DNA samples. 45 µl of master mix and 5 µl of DNA samples or the negative control (molecular-biology–grade water) were prepared and mixed in separately designated laboratory spaces. The negative control was used along with each 24-sample batch. Polymerase chain reaction (PCR) for DNA amplification was performed in the thermal cycler according to the protocol provided by the diagnostic kit's manufacturer. Amplification products were stored at 2-4°C until further processing.

Reverse Hybridisation

Before starting the test procedure, as stated in the manufacturer's instructions, reagents were brought to room tempera-

Table 1 Semi-quantitative interpretation of the test results

Bacterial species	Color intensity of the test strip bands representing detection scores 0-4				
	0	1	2	3	4
Aggregatibacter actinomycetemcomitans	$<10^3$ CFU/ml	10^3 CFU/ml	$<10^4$ CFU/ml	$<10^5$ CFU/ml	$>10^6$ CFU/ml
Porphyromonas gingivalis	$<10^4$ CFU/ml	10^4 CFU/ml	$<10^5$ CFU/ml	$<10^6$ CFU/ml	$>10^7$ CFU/ml
Prevotella intermedia					
Tannerella forsythia					
Treponema denticola					

Table 2 Distribution of sampled sites

Treatment	Second molars	First molars	Second premolars	First premolars	Canines	Lateral incisors	Central incisors
Control group (n)	10	5	9	10	16	25	21
Test group (n)	9	9	14	11	21	13	19
p	0.621	0.244	0.503	0.152	0.327	0.021	0.504

Mann-Whitney U-test for two independent groups.

ture (20-25°C) or heated to 45°C, and necessary dilutions were prepared. First, 20 µl of denaturation solution and 20 µl of amplified DNA sample were mixed and incubated at room temperature for 5 min. 1 ml of pre-warmed hybridisation buffer was added, and test strips were placed into each well containing denatured DNA samples. The prepared wells were incubated for 30 min at 45°C in a shaking water bath. After incubation, the hybridisation buffer was aspirated, and 1 ml of stringent wash solution was added to each well. The wells were incubated for 15 min at 45°C in the shaking water bath. The stringent wash solution was removed, and each strip was washed with 1 ml of rinse solution for 1 min on a shaking platform. 1 ml of diluted conjugate solution was added to each well and incubated for 30 min at room temperature on the shaking platform. Conjugate was removed, and each strip was washed for 1 min three times on a shaking platform: twice using rinse solution and once using distilled water. 1 ml of diluted substrate solution was added to each well and incubated protected from light and without shaking for 15 min. After test strip bands became clearly visible, they were briefly washed twice using distilled water, dried between two layers of absorbent paper, pasted on the provided evaluation sheet and stored protected from light.

Evaluation and Interpretation of Microbiological Results

First, developed test strips were inspected for effective and correct testing procedure by observing three control bands (conjugate control, hybridisation control, and amplification control). After making sure all three control bands were correctly developed, five bacterial species-specific bands were analysed by a semi-quantitative approach. According to the developed color intensity, 0, 1, 2, 3, or 4 points were assigned to each band. The color intensity of the bands is expressed semi-quantitatively as detection scores 0-4, which represent the number of CFUs/ml (Table 1).

Clinical Measurements

The following clinical parameters were measured to the nearest mm using a Williams periodontal probe (LM 51 ES, LM-Dental; Parainen, Finland) from the deepest site per quadrant at baseline, 3 and 6 months following the treatment:

- Bleeding on probing (BOP), defined as the percentage of sites positive for bleeding within 10 s after probing (%).
- Plaque index (PI), defined as the percentage of sites with visual plaque on the tooth surface (%).
- Probing depth (PD), measured in mm from the gingival margin to the bottom of the probed pocket.
- Recession (REC), measured in mm from the gingival margin to the cementoenamel junction or to the margin of a cervical restoration.
- Clinical attachment level (CAL), calculated by adding PD and REC at each site.

Blinding

Clinical measurements and microbial sampling were performed by a blinded calibrated examiner (U.M.D.), who was not aware in any of the cases of the type of treatment performed. To ensure blindness, the treatment procedures were performed by one experienced periodontist (E.R.). A third investigator

Table 3 Detection frequencies sorted by periodontopathogen (%)

Periodontopopathogen	Treatment strategy	Baseline	3 months	6 months
Aa	Control group	42.5	54.2	58.3
	Test group	45.8	29.2	33.3
Pg	Control group	75.0	58.3	75.0*
	Test group	87.5[ab]	41.7[a]	41.7*[b]
Tf	Control group	91.7[a]	62.5[a]	79.2
	Test group	83.3[ab]	54.2[a]	58.3[b]
Td	Control group	87.5[a]	58.3[a]	79.2*
	Test group	95.8[ab]	41.7[a]	33.3*[b]
Pi	Control group	58.3	29.2	45.8*
	Test group	45.8[ab]	20.8[a]	8.3*[b]

*$p < 0.05$, between-group differences (X^2 test). [a]$p < 0.05$, baseline and after 3 months (McNemar test); [b]$p < 0.05$, baseline and after 6 months (McNemar test).

(L.P.) performed microbiological analysis and was unaware of neither treatment procedures nor clinical measurements. I.N. processed coded data for statistical analysis.

Statistical Analysis

Statistical analysis was performed with the IBM SPSS 27 software package (IBM; Armonk, NY, USA). Data analysis was performed using the patient as the statistical unit. The difference in the distribution of sampled sites in terms of tooth group was examined using the Mann-Whitney U-test for two independent groups. For clinical changes at sampled sites, mean values per subject and per visit were calculated for each clinical parameter. The Shapiro-Wilk test was performed to assess whether clinical periodontal measures followed a normal distribution. If data followed a normal distribution, a paired-samples t-test was performed to evaluate before- and after-treatment comparisons within groups. If the data did not follow a normal distribution, the Wilcoxon signed-rank test was performed on related samples to assess before- and after-treatment comparisons within the groups. The between-group comparisons of measures were obtained by either the independent-samples t-test (if a parameter followed a normal distribution) or the Mann-Whitney test (if a specific measure followed a non-normal distribution).

Differences in detection frequency (0 = undetected and 1 = detected) between the control group and the test group at baseline and at 3 and 6 months were analysed using the X^2 test. The within-group changes were evaluated by McNemar test.

The changes of the detection frequency scores were recorded and classified into one of the following categories: 0: not detectable; or detectable with a score of 1, 2, 3 or 4 (Table1). Intragroup comparisons of detection scores of periopathogen species between the baseline and 3- and 6-month evaluation were performed using the Wilcoxon signed-rank test. The Mann-Whitney test was used for intergroup comparisons of detection scores for each timepoint. The significance level was set at 0.05.

RESULTS

All 48 patients completed the study. The distribution of sampled sites was equal in both groups in terms of tooth group, except for lateral incisors (Table 2).

Detection Frequency of Periodontopathogens

Table 3 displays the detection frequencies for each periodontopathogen at different time points in test and control groups. The results were expressed as the proportion of patients (%) positive for a given pathogen.

In the control group, after 3 months, statistically significant reductions were detected for T.f and T.d ($p < 0.05$), whereas after 6 months, the detected frequencies of the respective bacteria recovered to pretreatment levels and were comparable to the baseline values ($p > 0.05$). In the test group, statistically significant reductions were found for for P.g, T.f, T.d and P.i after 3 and 6 months ($p < 0.05$). The comparison of the test and control groups pointed to statistically significant differences in detection frequency of P.g ($p = 0.034$), T.d ($p < 0.01$) and P.i ($p = 0.02$) after 6 months, favouring the test group.

Changes of the Detection Scores of Periodontopathogens

Table 4 shows detection scores for A.a, P.g, T.f, T.d, and P.i at baseline, 3- and 6-month follow-ups.

At baseline, no statistically significant differences were observed between control and test groups in terms of detection scores of the investigated periodontal pathogenic species ($p > 0.05$). In the control group at 3 months, a statistically significant decrease in detection scores from baseline was found for P.g ($p = 0.013$), T.f ($p = 0.007$), T.d ($p = 0.003$) and P.i ($p = 0.012$). At 6 months, statistically significant reductions from baseline remained for P.g ($p = 0.039$) and T.f ($p = 0.048$). The test group at 3 months demonstrated a statistically significant decrease in detection scores from baseline for all investigated periopatho-

Table 4 Detection frequency scores for A.a, P.g, P.i, T.f, T.d at baseline, 3- and 6-month follow-up visits

Species	Timepoint	Detection score	Total, n (%)	Control group, n (%)	Test group, n (%)	p–value**
A.a	Baseline	0	22 (45.8)	9 (37.5)	13 (54.2)	0.174
		1	2 (4.2)	1 (4.2)	1 (4.2)	
		2	4 (8.3)	2 (8.3)	2 (8.3)	
		3	8 (16.7)	4 (16.7)	4 (16.7)	
		4	12 (25.0)	8 (33.3)	4 (16.7)	
	3 months	0	28 (58.3)	11 (45.8)	17 (70.8)	0.044
		1	2 (4.2)	1 (4.2)	1 (4.2)	
		2	6 (12.5)	4 (16.7)	2 (8.3)	
		3	6 (12.5)	2 (8.3)	4 (16.7)	
		4	6 (12.5)	6 (25.0)	0	
	*p–value		0.013	0.231	0.011	
	6 months	0	26 (54.2)	10 (41.7)	16 (66.7)	0.028
		1	4 (8.3)	1 (4.2)	3 (12.5)	
		2	4 (8.3)	3 (12.5)	1 (4.2)	
		3	6 (12.5)	3 (12.5)	3 (12.5)	
		4	8 (16.7)	7 (29.2)	1 (4.2)	
	*p–value		0.085	0.064	0.016	
P.g	Baseline	0	9 (18.8)	6 (25.0)	3 (12.5)	0.884
		1	1 (2.1)	–	1 (4.2)	
		2	2 (4.2)	1 (4.2)	1 (4.2)	
		3	11 (22.9)	4 (16.7)	7 (29.2)	
		4	25 (52.1)	13 (54.2)	12 (50.0)	
	3 months	0	24 (50.0)	10 (41.7)	14 (58.3)	0.099
		1	3 (6.3)	1 (4.2)	2 (8.3)	
		2	6 (12.5)	3 (12.5)	3 (12.5)	
		3	8 (16.7)	4 (16.7)	4 (16.7)	
		4	7 (14.6)	6 (25.0)	1 (4.2)	
	*p–value		<0.001	0.013	<0.001	
	6 months	0	20 (41.7)	6 (25.0)	14 (58.3)	0.006
		1	7 (14.6)	4 (16.7)	3 (12.5)	
		2	8 (16.7)	4 (16.7)	4 (16.7)	
		3	6 (12.5)	3 (12.5)	3 (12.5)	
		4	7 (14.6)	7 (29.2)	–	
	*p–value		<0.001	0.039	<0.001	
T.f	Baseline	0	6 (12.5)	2 (8.3)	4 (16.7)	0.846
		1	3 (6.3)	3 (12.5)	–	
		2	5 (10.4)	3 (12.5)	2 (8.3)	
		3	18 (37.5)	8 (33.3)	10 (41.7)	
		4	16 (33.3)	8 (33.3)	8 (33.3)	
	3 months	0	20 (41.7)	9 (37.5)	11 (45.8)	0.088
		1	8 (16.7)	1 (4.2)	7 (29.2)	
		2	8 (16.7)	5 (20.8)	3 (12.5)	
		3	10 (20.8)	7 (29.2)	3 (12.5)	
		4	2 (4.2)	2 (8.3)	–	
	*p–value		<0.001	0.007	<0.001	
	6 months	0	15 (31.3)	5 (20.8)	10 (41.7)	0.004
		1	10 (20.8)	3 (12.5)	7 (29.2)	
		2	9 (18.8)	4 (16.7)	5 (20.8)	
		3	12 (25.0)	10 (41.7)	2 (4.2)	
		4	2 (4.2)	2 (8.3)	–	
	*p–value		<0.001	0.048	<0.001	
T.d	Baseline	0	4 (8.3)	3 (12.5)	1 (4.2)	0.878
		1	10 (20.8)	4 (16.7)	6 (25.0)	
		2	22 (45.8)	11 (45.8)	11 (45.8)	
		3	12 (25.0)	6 (25.0)	6 (25.0)	
		4	–	–	–	
	3 months	0	24 (50.0)	10 (41.7)	14 (58.2)	0.125
		1	13 (27.1)	6 (25.0)	7 (29.2)	
		2	10 (20.8)	7 (29.2)	3 (12.5)	
		3	1 (2.1)	1 (4.2)	–	
		4	–	–	–	
	*p–value		<0.001	0.003	<0.001	
	6 months	0	21 (43.8)	5 (20.8)	16 (66.7)	<0.001
		1	13 (27.1)	6 (25.0)	7 (29.2)	
		2	12 (25.0)	11 (45.8)	1 (4.2)	
		3	2 (4.2)	2 (8.3)	–	
		4	–	–	–	
	*p–value		<0.001	0.083	<0.001	
P.i	Baseline	0	23 (47.9)	10 (41.7)	13 (54.2)	0.413
		1	5 (10.4)	4 (16.7)	1 (4.2)	
		2	4 (8.3)	–	4 (16.7)	
		3	12 (25.0)	8 (33.3)	4 (16.7)	
		4	4 (8.3)	2 (8.3)	2 (8.3)	
	3 months	0	36 (75.0)	17 (70.8)	19 (79.2)	0.399
		1	2 (4.2)	1 (4.2)	1 (4.2)	
		2	7 (14.6)	3 (12.5)	4 (16.7)	
		3	3 (6.3)	3 (12.5)	–	
		4	–	–	–	
	*p–value		<0.001	0.012	0.014	
	6 months	0	35 (72.9)	13 (54.2)	22 (91.7)	0.003
		1	4 (8.3)	3 (12.5)	1 (4.2)	
		2	5 (10.4)	4 (16.7)	1 (4.2)	
		3	4 (8.3)	4 (16.7)	–	
		4	–	–	–	
	*p–value		<0.001	0.091	0.003	

n: frequencies; *according to Wilcoxon tests for intragroup comparison of pathogen detection scores between successive timepoints; **according to Mann–Whitney tests for intergroup comparisons of pathogen detection scores for each timepoint.

Table 5 Clinical data of sampled sites (mean ± SD) at different time points

	Control group	Test group	p-value
PD (mm)			
Baseline	6.4 (1.0)	6.6 (1.2)	0.569[a]
After 3 months	3.3 (1.0)	2.5 (0.9)	0.02[a]
Baseline vs 3 months	<0.001[b]	<0.001[b]	
After 6 months	3.6 (0.8)	2.0 (0.8)	<0.001[a]
Baseline vs 6 months	<0.001[b]	<0.001[b]	
3 months vs 6 months	0.096[b]	0.003[b]	
CAL (mm)			
Baseline	6.4 (1.2)	6.4 (1.4)	0.844[a]
After 3 months	3.5 (1.0)	2.8 (1.2)	0.017[a]
Baseline vs 3 months	<0.001[b]	<0.001[b]	
After 6 months	3.9 (1.0)	2.3 (1.1)	<0.001[a]
Baseline vs 6 months	<0.001[b]	<0.001[b]	
3 months vs 6 months	0.084[b]	0.003[b]	
BOP (%)			
Baseline	92.1 (5.9)	94.2 (4.4)	0.429[a]
After 3 months	52.1 (14.2)	32.2 (14.6)	0.003[a]
Baseline vs 3 months	<0.001[b]	<0.001[b]	
After 6 months	59.4 (16.2)	19.2 (11.2)	<0.001[a]
Baseline vs 6 months	<0.001[b]	<0.001[b]	
3 months vs 6 months	0.072[b]	0.002[b]	
PI (%)			
Baseline	66.2 (22.1)	68.2 (11.2)	0.622[a]
After 3 months	21.2 (17.1)	19.2 (11.2)	0.002[a]
Baseline vs 3 months	<0.001[b]	<0.001b	
After 6 months	26.2 (21.3)	13.3 (6.8)	0.006[a]
Baseline vs 6 months	0.041[b]	<0.001[b]	
3 months vs 6 months	0.062[b]	0.003[b]	

[a] Statistical analysis using the Mann-Whitney test for two independent groups. [b] Paired-samples t-test for two dependent groups.

genic species: A.a ($p = 0.011$), P.g ($p < 0.001$), T.f ($p < 0.001$), T.d ($p < 0.001$) and P.i ($p = 0.014$). These results were maintained after 6 months: A.a ($p = 0.016$), P.g ($p < 0.001$), T.f ($p < 0.001$), T.d ($p < 0.001$) and P.i ($p = 0.003$). The intergroup analysis exhibited statistically significant differences in detection scores between control and test groups for A.a ($p = 0.044$) at the 3-month evaluation and for A.a ($p = 0.028$), P.g ($p = 0.006$), T.f ($p = 0.004$), T.d ($p < 0.001$) and P.i ($p = 0.003$) at the 6-month evaluation, favouring the test group.

Clinical Changes at Sampled Sites

Clinical changes at sampled sites are depicted in Table 5.

At baseline, no statistically significant differences were observed between test and control groups in any of the investigated clinical parameters ($p > 0.05$).

Regarding PD changes, both groups demonstrated statistically significant reductions in PD after 3 and 6 months; however, the difference between groups was statistically significant in favour of the test group at both timepoints ($p = 0.02$ and $p < 0.001$, respectively). Importantly, the PD change between 3- and 6- month follow-ups was statistically significant in the test group ($p = 0.003$), but did not demonstrate a statistically significant reduction in the control group ($p = 0.096$).

The intragroup comparisons pointed to a statistically significant gain in CAL in both groups at 3- and 6- month evaluations ($p < 0.05$), and intergroup analysis revealed statistically significant differences between groups at the respective timepoints ($p = 0.017$ and $p < 0.001$, respectively) in favour of the test group. The change in CAL between 3- and 6- months was statistically significant in the test group ($p = 0.003$), but did not demonstrate statistically significant improvements in the control group ($p = 0.084$).

Regarding changes in BOP, both study groups statistically significantly improved at 3 and 6 months compared to baseline ($p < 0.001$). The difference between groups was statistically significant at both the 3-month ($p = 0.003$) and the 6-month follow-up ($p < 0.001$). The change between 3- and 6-month evaluation was statistically significant in the test group ($p = 0.002$) but not ($p = 0.072$) in the control group.

In terms of PI, both groups showed statistically significant improvements at 3- and 6-month follow-ups compared to baseline ($p < 0.05$). The intergroup comparison revealed a statistically significant difference between groups in favour of the test group at 3 ($p = 0.002$) and 6 months ($p = 0.006$). The change between 3- and 6-month evaluation was statistically significant in the test group ($p = 0.003$) but not ($p = 0.062$) in the control group.

DISCUSSION

Recent studies indicated that clinical outcomes of non-surgical periodontal therapy can be improved by the adjunctive subgingival application of sodium hypochlorite/amino acids and cross-linked hyaluronic acid gels.[10,25,26] The present study investigated the microbiological impact of subgingivally delivered sodium hypochlorite/amino acids and cross-linked hyaluronic acid gels as adjuncts to same-day full-mouth subgingival debridement. To the authors' best knowledge, this is the first study to clinically evaluate the microbiological outcomes of this novel concept (i.e., "Clean and Seal") for non-surgical periodontal therapy.

Based on the present data, both treatment approaches (i.e., subgingival debridement and subgingival debridement in conjunction with sodium hypochlorite/amino acids and cross-linked hyaluronic acid gels) led to statistically significant microbiological shifts. However, these shifts exhibited different patterns between the test and control groups. In particular, after 3 months, both groups demonstrated statistically significant reductions in the detection frequency of T.f and T.d ($p < 0.05$), with the test group additionally showing a statistically significant reduction for P.i and P.g ($p < 0.05$). After 6 months, the detection frequency of T.f and T.d was comparable to baseline in the control group ($p > 0.05$), whereas statistically significant reductions ($p < 0.05$) compared to baseline were sustained in the test group for the respective bacterial species (T.f, T.d, P.i, and P.g). At this point, it is important to mention that the frequency of detecting A.a was unaffected by both treatment approaches ($p > 0.05$).

Similar findings have been reported in previous clinical studies on the effects of subgingival debridement on periodontal pathogens using molecular techniques, such as DNA probes and PCR amplification.[9,12,33] More specifically, only the levels of P.g, T.f, T.d and P.i statistically significantly decreased after non-surgical periodontal therapy,[9,12,33] while such changes were found to be statistically insignificant in terms of decreasing A.a.[9,33] These findings once again support the results from previous reports which failed to demonstrate the effectiveness of subgingival debridement alone in reducing A.a. levels.[35] Moreover, several studies have shown that statistically significant reductions in detection frequency of P.g, T.f, and T.d may be a characteristic feature of successful periodontal therapy.[7] Our observations align well with this statement, since at 3 months, a statistically significant reduction in detection frequency was found for P.g, T.f and T.d in the test group ($p < 0.05$) and for T.f and T.d in the control group ($p < 0.05$), whereas after 6 months, the reductions remained stable for P.g, T.f and T.d only in the test group ($p < 0.05$).

Regarding the changes of detection scores, after 3 months, both study groups demonstrated statistically significant reductions of T.f, T.d, P.g, and P.i ($p < 0.05$) compared to baseline, while a statistically significant reduction of A.a was only observed in the test group ($p = 0.001$). At 6 months, a statistically significant reduction compared to baseline persisted for P.g and T.f in the control group ($p < 0.001$). However, in the test group, the reduction remained statistically significant for all investigated periodontal pathogenic species compared with baseline ($p < 0.05$). These findings corroborate those obtained in a recent 12-month randomised controlled clinical trial[24] that evaluated changes in detection scores for five periodontal pathogenic species and pointed towards statistically significant benefits of the adjunctive application of sodium hypochlorite/amino acids gel to subgingival debridement in reducing the detection scores of P.g ($p = 0.015$) and T.f ($p = 0.004$). However, the levels of A.a remained unchanged compared with baseline ($p = 0.098$).[24] Moreover, another clinical trial, which investigating the presence or absence of six target microorganisms in pockets treated with either ultrasonic instrumentation (control) or ultrasonic instrumentation supplemented with sodium hypochlorite/amino acid gel (test), found statistically significant reductions in T.f from baseline to day 7 ($p < 0.05$) and in T.d from baseline to month 4 ($p < 0.05$) in the test group.[19]

The differences observed in the present analysis between the test and control groups regarding detection frequencies and changes in detection scores may be attributed to the additive antimicrobial effects of sodium hypochlorite/amino acid and cross-linked hyaluronic acid gels.[17,23] Based on previous findings from in-vitro and animal experiments, it may be hypothesised that the ability of sodium hypochlorite/amino acids to facilitate mechanical debridement and biofilm removal may lend additional support to xHyA in expressing its bacteriostatic and wound healing properties.[17,23,30-31] In fact, as pointed out by the numerous clinical studies,[4,8,13,20] mechanical debridement alone has only limited efficacy in eradicating all bacteria, particularly keeping in mind that bacteria may reside in soft tissues, root surface irregularities and dentinal tubules.[22]

The present work also analysed microbial samples taken from treated patients from our previous randomised clinical trial;[26] the results are reported here. We therefore show that previously reported clinical data[26] align well with the microbiological outcomes reported in this paper. When interpreting the data, it is important to point out that the obtained microbiological findings correspond well with the clinical outcomes assessed after 3 and 6 months after treatment. In particular, after 3 months, both study groups demonstrated statistically significant improvements in PD, BOP, PI reductions and CAL gain with a statistically significant difference in favour of the test group. An interesting finding was that after 6 months, the test group exhibited gradual and significant clinical improvements in PD, CAL, BOP, and PI compared to the 3-month evaluation. In contrast, the results in the control group remained unchanged or showed signs of relapse.

Taken together, these findings demonstrate that the microbiological benefits of sodium hypochlorite/amino acids and cross-linked hyaluronic acid gels were sustained over a 6-month period, indicating a long-term microbiological effect. Furthermore, a connection between clinical and microbiological status can be confirmed; however, it remains unclear whether a decrease in subgingival microbiota led to an improvement in clinical conditions or vice versa. When interpreting the results, the question arises as to what extent each of the adjunctive substances used (i.e., sodium hypochlorite/amino acids and cross-linked hyaluronic acid) contributed to the additional microbiological improvements observed in the test group. In this respect, it is important to emphasise that the present study used the combination of the two materials as a

single concept. Therefore, further studies are needed to better understand the separate and combined effects of the two components on the clinical and microbiological outcomes.

CONCLUSION

The microbiological results of the present study support the adjunctive subgingival application of sodium hypochlorite/amino acid and xHyA to subgingival debridement in the treatment of periodontitis.

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RESEARCH

Clinical evaluation of sodium hypochlorite/amino acids and cross-linked hyaluronic acid adjunctive to non-surgical periodontal treatment: a randomized controlled clinical trial

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Abstract

Objectives To compare the clinical outcomes obtained with either mechanical subgingival debridement in conjunction with a sodium hypochlorite and amino acids containing gel followed by subsequent application of a cross-linked hyaluronic acid gel (xHyA) gel, or with mechanical debridement alone.

Materials and Methods Fourty-eight patients diagnosed with stages II-III (Grades A/B) generalised periodontitis were randomly treated with either scaling and root planing (SRP) (control) or SRP plus adjunctive sodium hypochlorite/amino acid and xHyA gels (test). The primary outcome variable was reduction of probing depth (PD), while changes in clinical attachment level (CAL), bleeding on probing (BOP) and plaque index (PI) were secondary outcomes. The outcomes were assessed at baseline, at 3 and 6 months following therapy.

Results All patients completed the 6 months evaluation. At 6 months, the test group showed statistically significantly better results in terms of mean PD reduction (2.9 ± 0.4 vs 1.8 ± 0.6 mm, $p < 0.001$). Similarly, mean CAL gain was statistically higher in the test group compared to the control one (test: 2.6 ± 0.5 vs control: 1.6 ± 0.6 mm, $p < 0.001$). Mean BOP decreased from $81.8 \pm 16.2\%$ to $48.9 \pm 14.5\%$ in control ($p < 0.001$) and from $83.2 \pm 15.5\%$ to $17.6 \pm 11.5\%$ in test ($p < 0.001$) groups with a statistically significant difference favouring the test group ($p < 0.001$). Mean PI scores were reduced statistically significantly in both groups (from $38.8 \pm 26\%$ to $26.5 \pm 20.5\%$ in control ($p = 0.039$) and from $60.6 \pm 10.9\%$ to $12.7 \pm 8.9\%$ in test group ($p < 0.001$)), with a statistically significant difference between the groups ($p < 0.001$). The number of moderate pockets (4–6 mm) were reduced from 1518 (41.2%) to 803 (22.6%) in the control and from 1803 (48.6%) to 234 (7.7%) in the test group with a statistically significant difference between the groups ($p < 0.001$), while the number of deep pockets (≥ 7 mm) changed from 277 (7.6%) to 35 (1.0%) in the control and from 298 (8.7%) to 4 (0.1%) in test group ($p = 0.003$).

Conclusion Within their limits the present data indicate that: a) both treatments resulted in statistically significant improvements in all evaluated clinical parameters, and b) the adjunctive subgingival application of sodium hypochlorite/amino acid and xHyA to SRP yielded statistically significantly higher improvements compared to SRP alone.

Clinical relevance The combination of sodium hypochlorite/amino acid and xHyA gels to subgingival mechanical debridement appears to represent a valuable approach to additionally improve the outcomes of non-surgical periodontal treatment. Clinical Trial Registration Number NCT04662216 (ClinicalTrials.gov).

Keywords Periodontitis · Non-surgical periodontal therapy · Cross-linked hyaluronic acid · Sodium hypochlorite/amino acids

Introduction

Periodontitis is a chronic multifactorial inflammatory disease caused by dysbiotic dental plaque biofilms with the formation of an inflammatory infiltrate that contributes to destruction of connective tissue attachment to the tooth, alveolar bone resorption and may result in tooth loss [1–5]. In case of periodontitis a disruption of the normal function of the healthy subgingival plaque biofilm with concomitant disruption to its functional properties in relation to innate defense surveillance and tissue maintenance, leading to excessive, deregulated inflammation and tissue destruction is observed [6, 7].

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Primary clinical features of periodontitis include the loss of periodontal tissue support, which manifests through clinical attachment loss and radiographically assessed alveolar bone loss with the presence of gingival bleeding and periodontal pockets [5]. The recently published clinical practice guidelines for treating stage I–III periodontitis concluded that cause-related therapy is aimed at reducing/eliminating the subgingival biofilm and calculus by means of subgingival instrumentation, which may include the adjunctive application of physical or chemical agents [8].

Recent systematic reviews have provided some evidence indicating that adjunctive aids, in conjunction with mechanical debridement, might enhance the outcomes of non-surgical periodontal therapy [9–12]. More recently, findings from *in vitro* experiments have shown, that a sodium hypochlorite gel has a softening effect on the extracellular biofilm matrix which in turn, may facilitate its mechanical removal. It has been shown that the effect of sodium hypochlorite/amino acid gel is due to its active part, the chloramine, which forms following the chlorine transfer of sodium hypochlorite to the amine functions of the added amino acids [13]. Amino acids act like a buffer and provide protection to soft tissues. The high pH (11) of this formulation has a softening effect on the calculus, which makes the cleaning process easier [14]. Therefore, it may be anticipated that during subgingival debridement treatment, both the mechanical and chemical components act synergistically to disrupt the hard and soft biofilm which in turn, may facilitate granulation tissue removal [13, 14]. In this respect, positive clinical effects of a sodium hypochlorite gel were reported in studies treating residual periodontal pockets [15, 16], peri-implant mucositis [17] and peri-implantitis [14].

HA is a naturally occurring biodegradable polymer that is responsible for several structural properties of tissues as a component of the extracellular matrix [18]. Several studies have provided evidence indicating that HA plays an important role in wound healing, supports scarless wound-healing, promotes angiogenesis and has a bacteriostatic effect in surgical wounds [19–22]. When used during periodontal surgery, HA has been shown to promote periodontal regeneration in intrabony, recession and furcation defects [23–25]. Clinical studies revealed that HA may represent a valuable constituent to mechanical debridement (i.e., scaling and root planing), thus resulting in statistically significant clinical improvements, evidenced by reduction in probing depth (PD), gain of clinical attachment (CAL) and improved bleeding on probing (BOP) values, compared to scaling and root planing alone [26–29].

Recently, a novel concept consisting of enhancing biofilm removal during nonsurgical therapy by means of a sodium hypochlorite/amino acids followed by application of a cross-linked hyaluronic acid gel (xHyA) gel was suggested as a novel strategy to improve the outcomes of nonsurgical periodontal therapy [30, 31]. Results from two case series have shown statistically significant clinical improvements compared to baseline following scaling and root planing in conjunction with sodium hypochlorite/amino acid and xHyA, thus suggesting that this strategy may represent a valuable novel strategy in non-surgical periodontal treatment.

However, to the best of our knowledge, at present no randomized controlled clinical trials have evaluated the potential clinical relevance of this novel concept as compared to mechanical debridement alone.

Therefore, the aim of this randomized controlled clinical study was to compare the clinical outcomes obtained with either mechanical subgingival debridement in conjunction with sodium hypochlorite/amino acid gel followed by subsequent application of xHyA, or with mechanical debridement alone.

Material and methods

Study design

This study was conducted as a 6-months prospective, examiner-blind, randomized controlled clinical trial with a parallel design. The study was performed according to CONSORT guidelines for randomized controlled clinical trials (http://www.consort-statement.org/) [32]. Ethical permission was issued by the Regional Biomedical Research Ethics Committee (No. BE-2–87). Prior to participation, all patients signed a written informed consent form. After signing the informed consent form, the patients were randomly assigned to the control or test groups (allocation ratio 1:1). The study was conducted between September 2019 and January 2022. In addition, the study protocol was registered at ClinicalTrials.gov, NCT04662216.

Study population

All patients included in the study were enrolled and treated at the Department of Dental and Oral Pathology at the Lithuanian University of Health Sciences in Kaunas, Lithuania.

Inclusion criteria:

- Males and females ≥ 18 years old.
- Periodontitis stages II–III, grades A/B, generalised [5].
- Good general health (i.e., absence of systemic diseases and no intake of medication which may affect periodontal health).
- Presence of at least 20 teeth (wisdom teeth excluded).
- Absence of removable dentures.
- Patients willing to provide written informed consent and willing to complete the 6-month study follow-up.

Exclusion criteria:

- Patients already included in other clinical trials.
- Smokers.
- Periodontal treatment during the last 12 months.
- Antibiotic treatment 3 months prior to the start of the trial.
- Antibiotic prophylaxis required for dental treatment.
- Ongoing medication that may affect the clinical features of periodontitis.
- Pregnant/lactating.
- Allergies to sodium hypochlorite

Sample size calculation

At the start of the study, a significance level of $\alpha = 0.05$, a relevant average difference in PD of 1 mm between study groups with a standard deviation of 1 mm and a power (1—α) of at least 0.8 were set to calculate the minimum number of necessary cases (at least 20 per group). Assuming any possible dropouts during the study period, the number of patients was increased to 24 in each group. A power calculation at the end of the study with the given number of cases yielded a power of 99.6%.

Periodontal treatment

Baseline periodontal measurements were obtained 2 weeks prior to the treatment, which was followed by professional supragingival tooth cleaning and individual oral hygiene instructions for all included patients. These treatments included manual toothbrushes and interdental brushes. All patients were provided the same type of toothpaste (Elmex Enamel Protection, Gaba GmbH, Germany) and tooth (CS 5460, Curaprox, Curaden, Switzerland) and interdental (TePe, Tepe Mundhygienprodukten, Sweden) brushes. Oral hygiene instructions were reinforced at each follow-up visit, but no further treatment was rendered.

Two weeks later, under local anaesthesia, subjects in the control group underwent full-mouth SRP performed with ultrasonic (Satelec/Acteon suprasson newtron ultrasonic scaler) and hand instruments (LM SharpDiamond 1/2, 7/8, 11/12, 13/14 SD mini Gracey and Gracey curettes, LM Dental™, Finland). Subsequently, all teeth were polished using a low-abrasive paste (Lunos Super Soft, RDA < 5, Dürr Dental, Germany). Mechanical debridement took on average 3.5 h per patient.

In the test group, full-mouth SRP was performed as follows: in all pockets with PD ≥ 4 mm a sodium hypochlorite/amino acid gel (Perisolv®, Regedent AG, Zürich, Switzerland) was instilled into the pockets and kept there for 60 s before subgingival instrumentation. Subgingival instrumentation was carried out with the same ultrasonic and hand instruments and the application of sodium hypochlorite/amino acid gel was repeated until the instrumentation was considered sufficient (i.e., for a total of 2–3 times) (Fig. 1). All treatments were performed with magnifying glasses (4.5X – Ergo Advanced, Univet, Rezzato BS, Italy) and sufficient instrumentation was attained when root surfaces exhibited smooth surfaces upon probing with an explorer probe (Explorer-Periodontal Probe 8-520B, LM Dental™, Finland). Following SRP, a mixture of natural and cross-linked hyaluronic acid (high molecular) gel (Hyadent® BG, Regedent AG, Zürich, Switzerland) was instilled in the pockets using a blunt needle (Fig. 2).

Clinical measurements

The following clinical parameters were assessed using a Williams periodontal probe to the nearest mm (LM 51 ES, LM-Dental™, Finland) on all teeth at 6 sites per tooth (i.e., mesio-buccal (mb), mid-buccal (b), disto-buccal (db), mesio-oral (mo), mid-oral (o) and disto-oral (do)) at baseline (T0), 3 months (T1) and 6 months (T2) following the treatment:

- Bleeding on probing (BOP), defined as the percentage of sites positive to bleeding within 10 s after probing (%). BOP was assessed for treated sites (PD ≥ 4 mm) and full mouth (FMBOP).
- Plaque index (PI), defined as the percentage of sites with visual plaque on the tooth surface (%). PI was assessed at treated sites (PD ≥ 4 mm) as well as the full mouth (FMPI).
- Probing depth (PD), measured in millimetres from the gingival margin to the bottom of the probed pocket.
- Recession (REC), measured in millimetres from the gingival margin to the cemento-enamel junction or to the margin of a cervical restoration.

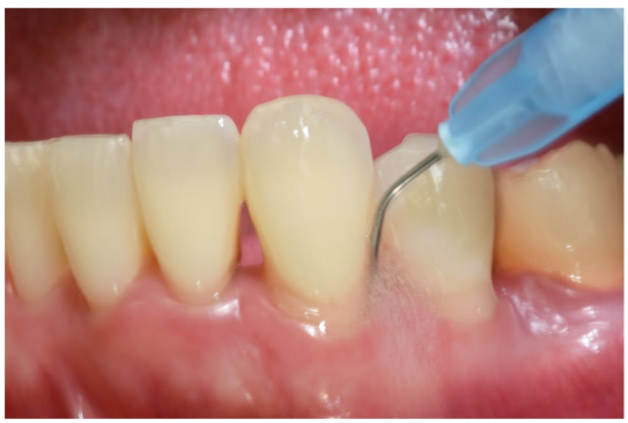

Fig. 1 Application of sodium hypochlorite/amino acid gel to the periodontal pocket

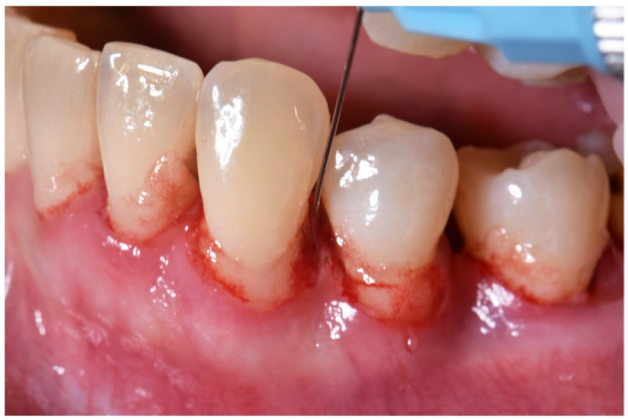

Fig. 2 Application of a mixture of natural and cross-linked hyaluronic acid (high molecular)to the periodontal pocket

- Clinical attachment level (CAL), calculated by adding PD and REC at each site.

At each visit, the clinical examiner had to record possible complications or adverse events related to the tested materials or study interventions, as well as those reported by study subjects.

Outcomes

For data analysis, PDs were subdivided into two categories: moderate (PD 4–6 mm) and deep (PD ≥ 7 mm). The primary outcome variable was the mean PD change from baseline to 6 months in moderate sites. Secondary outcome variables included PD change in deep pockets at 6 months, as well as CAL changes in moderate and deep sites. In addition, mean BOP and PI changes from baseline to 6 months in all treated sites (PD ≥ 4 mm) and the full mouth were evaluated.

Blinding

Clinical measurements and initial supragingival tooth cleaning were performed by a blinded calibrated examiner (U.M.D.), who was not aware in any of the cases of the type of treatment performed. All recordings were made without access to previous measurements to avoid bias.

To ensure blindness, the treatment procedures were performed by one experienced periodontist (E.R.).

The patients were not aware to which group they had been assigned. Periodontal treatment was performed in a sterile field (face drapes were used) to eliminate the possibility for patients to observe the procedure.

A third investigator (I.N.), unaware of the type of treatment performed, processed coded data for statistical analysis.

Randomization and allocation concealment

Forty-eight patients were randomized into two treatment groups. A computer-generated randomization table was created. Patients were assigned unique numbers from 1 to 48, and 2 sets of randomized numbers were generated (24 for control group subjects and 24 for test). Allocation concealment was performed using sealed envelopes to be opened before periodontal treatment. The generation of the random sequence allocation and the assignment of participants to interventions were performed by the investigator, distinct from the clinical examiner and the clinician who performed the treatment.

Calibration

Five patients, not related to the study, each diagnosed with periodontitis stages II–III [5], were used to calibrate the examiner (U.M.D.). The examiner was asked to evaluate PD, REC, CAL, BOP and PI at 6 sites per tooth on 2 separate appointments, 48 h apart. Calibration was accepted if measurements at baseline and at 48 h were equal to the millimetre at > 90% level. The examiner was not aware of the procedure to be performed.

Statistical analysis

Statistical analysis was performed with the IBM SPSS 27 software package (IBM Corp.). Data analysis was performed using the patient as the statistical unit. For all clinical parameters, mean values per subject and per visit were calculated. In particular, PD and CAL of moderate pockets at baseline and at 3- and 6-month follow-ups were obtained by averaging PDs and CALs in moderate sites for each patient at baseline, 3- and 6-month follow-ups. Similarly, per-patient PD and CAL of deep pockets at baseline (and at 3 and 6 months) were obtained by averaging PD and CAL values in deep sites for each patient at baseline, 3 and 6 months. Per-patient BOP and PI were obtained by calculating a percentage share of tooth sites with BOP and plaque for each patient by classifying pockets by baseline PD (all treated sites with PD ≥ 4 mm and the full mouth).

The Shapiro–Wilk test was performed to assess whether clinical periodontal measures followed a normal distribution. If data followed a normal distribution, a paired-samples *t* test was performed to evaluate before- and after-treatment comparisons within groups. If the data did not follow a normal distribution, the Wilcoxon signed rank test was performed on related samples to assess before- and after-treatment comparisons within the groups. The between-group comparisons of measures were obtained by either the independent-samples *t* test (if a parameter followed a normal distribution) or the Mann–Whitney test (if a specific measure followed a non-normal distribution). The significance level was set at 0.05.

Results

Participant flow

All 48 patients completed the study. Each treatment group (SRP or SRP + sodium hypochlorite/amino acid + xHyA) consisted of 24 randomly selected patients. A flowchart of the study is depicted in the CONSORT flow diagram (Fig. 3). In all subjects, healing was uneventful. No adverse effects of sodium hypochlorite/amino acid and xHyA were observed during the study period.

Baseline characteristics

Clinical and demographic baseline characteristics of the 48 participants are shown in Table 1. The baseline examination revealed that the two study groups showed similar characteristics for PD, CAL, bleeding (BOP and FMBOP) and plaque scores with no significant differences between the groups (except for PI and FMPI) (Table 1A). Furthermore, regarding the number of type of treated teeth, no statistically significant differences were observed between control and test groups (Table 1B).

Effect on clinical parameters

PD changes during the study period were analysed for different pocket categories: mean moderate (4–6 mm) and mean deep (PD ≥ 7 mm) pockets. Data is presented in Table 2.

In mean moderate pockets, the baseline values did not reveal a statistically significant difference between control and test groups (4.8 ± 0.2 and 4.7 ± 0.2, respectively, $p = 0.417$). Both groups showed statistically significant improvements at 3 and 6 months compared to baseline ($p < 0.001$); however, statistically significantly higher reductions were observed in favour for the test group at both points in time ($p < 0.001$) (Table 2). The change of PD between 3 and 6 months differed statistically significantly between groups in favour for the test group ($p = 0.002$) (Fig. 4).

Baseline PD values in mean deep pockets category were not statistically significantly different between control and test groups (8.0 ± 0.7 and 8.2 ± 0.9, respectively, $p = 0.443$). Both groups reached statistically significant improvements at 3 and 6 months compared to baseline ($p < 0.001$); however, PD reduction in the test group was statistically significantly higher compared to the control group at both follow-ups ($p < 0.001$) (Table 2). The change between 3 and 6 months did not differ between the groups ($p = 0.096$) (Fig. 5).

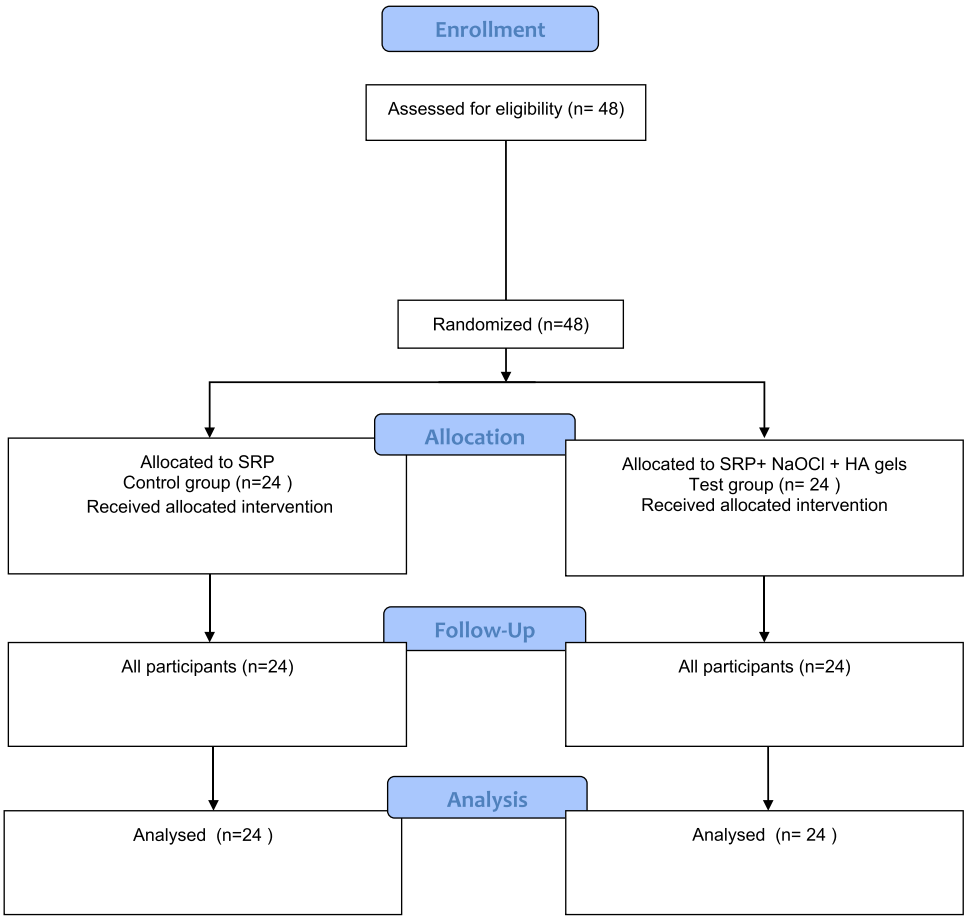

Fig. 3 CONSORT flow diagram of participant recruitment

Table 1 Clinical and demographic characteristics of sample population at the baseline

A. Characteristics of sample population at the baseline

	SRP ($N=24$)	SRP + NaOCl + HA ($N=24$)	*P* value
Age (years)	49.3 ± 11.2	47.3 ± 10.7	0.53[a], n.s
Gender, *n* (%)			
Males	7 (29.2)	6 (25)	0.745[b],
Females	17 (70.8)	18 (75)	n.s
Periodontitis stage, *n* (%)			
Stage II	16 (66.7)	17 (70.8)	0.134[b],
Stage III	8 (33.3)	7 (29.2)	n.s
Grade A	13 (54.2)	12 (50.0)	
Grade B	11 (45.8)	12 (50.0)	0.242[b], n.s
PD (mm)	5.3 ± 0.6	5.2 ± 0.4	0.592[c], n.s
CAL (mm)	5.5 ± 0.5	5.6 ± 0.6	0.546[c], n.s
PI (%)	38.8 ± 26	60.6 ± 10.9	**0.002[c]**
BOP (%)	81.8 ± 16.2	83.2 ± 15.5	0.687[c], n.s
FMPI (%)	35.7 ± 23.7	52.9 ± 11.4	**0.003[c]**
FMBOP (%)	68.9 ± 20.3	76.5 ± 18.2	0.184[c], n.s

B. Distribution of treated teeth

Treatment	Second Molars	First Molars	Second Premolars	First Premolars	Canines	Lateral Incisors	Central incisors
Control group (*n*)	88	84	89	91	94	94	95
Test group (*n*)	86	90	86	89	96	96	96
p	0.549	0.187	0.505	0.682	0.153	0.153	0.317

BOP – bleeding on probing; CAL – clinical attachment level; FMBOP – full-mouth bleeding on probing; FMPI – full-mouth plaque index; PD – probing depth; PI – plaque index

n.s. not significant

[a] Independent-samples *t* test

[b] Fisher's exact test for the 2 × 2 table, sex by group (SRP, SRP + NaOCl + xHyA)

[c] Mann–Whitney U test for two independent groups

Mann–Whitney U test for two independent groups

Table 2 PD (mean (SD)) at sites with moderate (4–6 mm) and deep (≥ 7 mm) pockets

	Control group ($n=24$)	Test group ($n=24$)	*p* value
Moderate pockets (4–6 mm)			
Baseline	4.8(0.2)	4.7(0.2)	0.417[a]
After 3 months	2.9(0.7)	2.2(0.4)	< 0.001[a]
Baseline vs. 3 months	< 0.001[b]	< 0.001[b]	< 0.001[a]
After 6 months	3.0(0.6)	1.8(0.4)	
Baseline vs. 6 months	< 0.001[b]	< 0.001[b]	
Deep pockets (≥ 7 mm)			
Baseline	8.0(0.7)	8.2(0.9)	0.443[a]
After 3 months	4.4(1.4)	2.9(1.1)	< 0.001 [a]
Baseline vs. 3 months	< 0.001[b]	< 0.001[b]	< 0.001
After 6 months	4.3(1.0)	2.4(1.0)	
Baseline vs. 6 months	< 0.001[b]	< 0.001[b]	

[a] Statistical analysis by Student's t test for two independent groups

[b] Paired Samples T Test for two dependent groups

CAL changes in mean moderate and mean deep pockets are reported in Table 3.

At baseline, in mean moderate pockets group, the CAL values were slightly higher in the control group (4.8 ± 0.3 mm) compared to the test group (4.6 ± 0.2 mm; $p=0.026$). Both groups reached significant improvements at 3 and 6 months compared to baseline ($p<0.001$); however, a statistically significant difference between groups was observed in favour of the test group at both points in time ($p<0.001$) (Table 3). Mean CAL change between the 3- and 6-month follow-ups was statistically significantly different between the groups in favour for the test group ($p=0.004$) (Fig. 6).

In mean deep pockets baseline, CAL values were not statistically significantly different and measured 7.9 ± 0.6 mm in the control group and 8.1 ± 0.7 mm in the test group ($p=0.412$), respectively. Both groups reached statistically significant improvements at both follow-ups, compared to baseline ($p<0.001$); however, statistically significantly

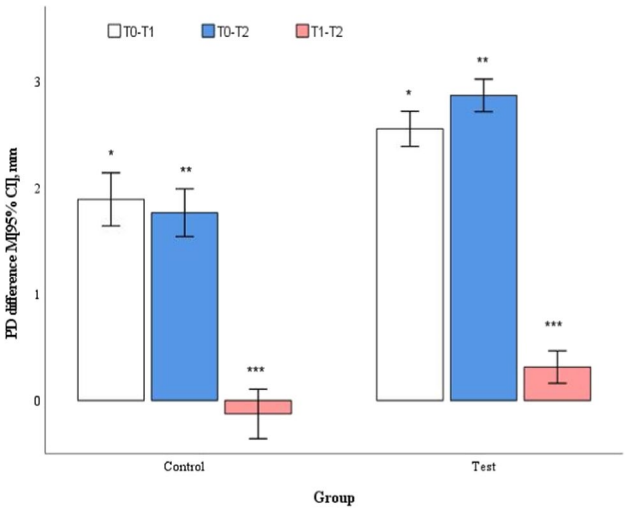

Fig. 4 Mean changes in PD in moderate pockets (4-6 mm) at different time points. $^{*,**}p<0.001$, $^{***}p=0.002$ by Student's t test for two independent groups. T0 – baseline; T1 – 3 months follow-up; T2 – 6 months follow-up

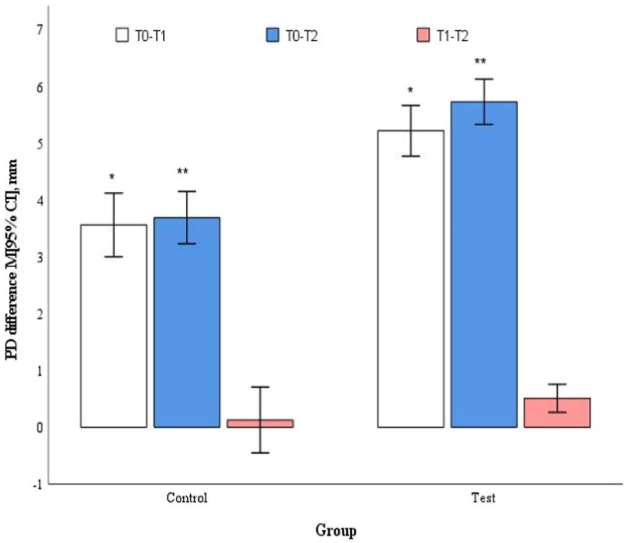

Fig. 5 Mean changes in PD in deep pockets (≥7 mm) at different study time points. $^{*,**}p<0.001$, by Student's t test for two independent groups. T0 – baseline; T1 – 3 months follow-up; T2 – 6 months follow-up

Table 3 CAL (mean ± SD) at sites with moderate (4–6 mm) and deep (≥7 mm) pockets

	Control group ($n=24$)	Test group ($n=24$)	p value
Moderate pockets (4–6 mm)			
Baseline	4.8(0.3)	4.6(0.2)	0.026[a]
After 3 months	3.1(0.8)	2.4(0.6)	<0.001[a]
Base vs. 3 months	<0.001[b]	<0.001[b]	<0.001[a]
After 6 months	3.1(0.7)	2.0(0.5)	
Base vs. 6 months	<0.001[b]	<0.001[b]	
Deep pockets (≥7 mm)			
Baseline	7.9(0.6)	8.1(0.7)	0.412[a]
After 3 months	4.5(1.2)	3.2(1.4)	0.002 [a]
Base vs. 3 months	<0.001[b]	<0.001[b]	<0.001 [a]
After 6 months	4.6(1.0)	2.8(1.3)	
Base vs. 6 months	<0.001[b]	<0.001[b]	

[a] Statistical analysis by Student's t test for two independent groups

[b] Paired Samples T Test for two dependent groups

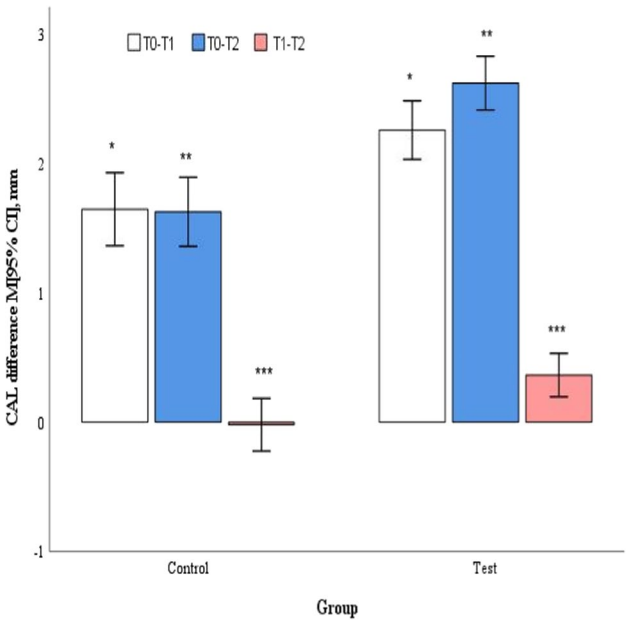

Fig. 6 Mean changes in CAL in moderate pockets (4-6 mm) at different study time points. $^{*,**}p<0.001$, $^{***}p=0.004$ by Student's t test for two independent groups. T0 – baseline; T1 – 3 months follow-up; T2 – 6 months follow-up

better improvements were achieved in favour for the test group ($p<0.001$) (Table 3). Mean CAL change between 3- and 6-month follow-up did not show a statistically significant difference between the groups ($p=0.077$) (Fig. 7).

BOP changes were evaluated for treated sites (PD ≥ 4 mm) and full mouth (FMBOP).

Regarding full-mouth measurements, baseline FMBOP values were similar in test (76.5 ± 18.2%) and control (68.9 ± 20.3%) groups ($p=0.184$). Both study groups reached significant improvements at 3 and 6 months compared to baseline ($p<0.001$). The difference between groups was not statistically significant at the 3-month follow-up ($p=0.06$) but reached a statistically significant difference in favour for the test group at the 6-month follow-up ($p<0.001$) (Table 4).

The analysis of treated pockets (PDs ≥ 4 mm) revealed no statistically significant difference in baseline BOP values between test and control groups ($p=0.687$). Although both

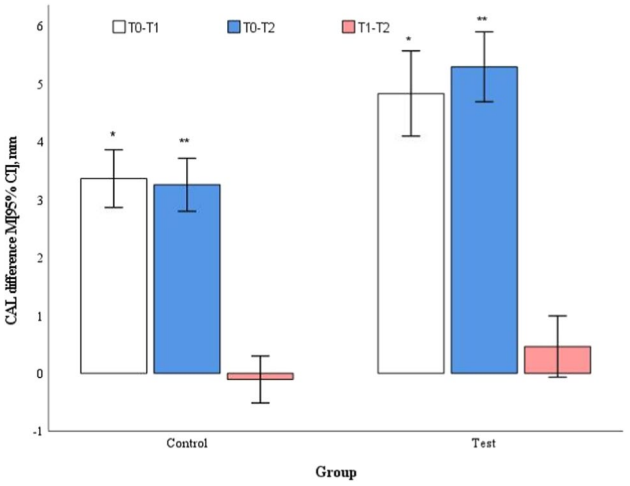

Fig. 7 Changes in CAL in deep pockets (≥7 mm) at different study time points. $^{*}p=0.002$, $^{**}p<0.001$, by Student's t test for two independent groups. T0 – baseline; T1 – 3 months follow-up; T2 – 6 months follow-up

groups showed statistically significant improvements at 3- and 6-month follow-ups compared to baseline ($p<0.001$), the reduction of BOP was statistically significantly better in the test group compared to the control group at both points in time ($p=0.018$ and $p<0.001$, respectively) (Table 4).

PI changes were evaluated for treated sites (PD ≥ 4 mm) and the full mouth (FMPI).

Baseline FMPI values were higher in the test group (52.9 ± 11.4%) than in the control one (35.7 ± 23.7%) ($p=0.003$). However, both groups showed significant improvements at 3- and 6-month follow-ups compared to baseline ($p<0.001$). The intergroup comparison revealed a statistically significant difference between groups in favour for the test group at 6 months ($p=0.006$) (Table 5).

A similar pattern was observed in the analysis for PI at treated pockets. In particular, higher PI (%) values were reported in the test group than the control group ($p=0.002$). Both study groups showed statistically significant improvements at 3- and 6-month evaluations, compared to baseline ($p<0.001$). No statistically significant difference was observed between groups at the 3-month evaluation ($p=0.714$), whereas at the 6-month examination, the reduction in PI was statistically significantly greater in the test group ($p=0.018$) (Table 5).

Analysis of frequency distributions of shallow, medium, and deep pockets

Additionally, the analysis of frequency distribution of shallow (1–3 mm), medium (4–6 mm) and deep (≥7 mm) sites at baseline, 3 and at 6 months was performed (Table 6). At baseline, subjects in the control group had 1518 (41.2%) sites with moderate pockets (4-6 mm) and test group 1803 (48.6%) sites, respectively. At 6 months this number reduced to 803 (22.6%) in control and 234 (7.7%) sites in the test

Table 4 BOP (%) at treated sites (PD ≥ 4 mm) and full mouth (mean ± SD)

	BOP		*P* value	FMBOP		*P* value
	Control ($n=24$)	Test ($n=24$)		Control ($n=24$)	Test ($n=24$)	
Baseline	81.8 ± 16.2	83.2 ± 15.5	0.687[a]	68.9 ± 20.3	76.5 ± 18.2	0.184[a]
After 3 months	39.1 ± 15.9	28.3 ± 14.6	**0.018**[a]	33.3 ± 13.7	25.9 ± 12.3	0.06[a]
Baseline vs 3 months	**< 0.001**[b]	**< 0.001**[b]		**< 0.001**[b]	**< 0.001**[b]	
After 6 months	48.9 ± 14.5	17.6 ± 11.5	**< 0.001**[a]	40.8 ± 13.8	15.6 ± 9.9	**< 0.001**[a]
Baseline vs 6 months	**< 0.001**[b]	**< 0.001**[b]		**< 0.001**[b]	**< 0.001**[b]	

[a] Statistical analysis by Student's t or Mann–Whitney test for two independent groups

[b] Wilcoxon Signed Ranks Test for two dependent groups

Table 5 PI (%) at treated sites (PD ≥ 4 mm) and full mouth (mean ± SD)

	PI		*P* value	FMPI		*P* value
	Control	Test		Control	Test	
Baseline	38.8 ± 26	60.6 ± 10.9	**0.002**[a]	35.7 ± 23.7	52.9 ± 11.4	**0.003**[a]
After 3 months	20.3 ± 16.7	18.8 ± 11.4	0.714[a]	19.3 ± 15.0	17.1 ± 9.7	0.893[a]
Baseline vs 3 months	**< 0.001**[b]	**< 0.001**[b]		**< 0.001**[b]	**< 0.001**[b]	
After 6 months	26.5 ± 20.5	12.7 ± 8.9	**0.018**[a]	23.5 ± 16.6	11.2 ± 7.9	**0.006**[a]
Baseline vs 6 months	**0.039**[b]	**< 0.001**[b]		**< 0.001**[b]	**< 0.001**[b]	

[a] Statistical analysis by Student's t or Mann–Whitney test for two independent groups

[b] Wilcoxon Signed Ranks Test for two dependent groups

group with a statistically significant difference between the groups ($p < 0.001$). Similarly, the number of deep pockets (≥ 7 mm) changed from 277 (7.6%) to 35 (1.0%) in control and from 298 (8.7%) to 4 (0.1%) in test at 6 months evaluation with a statistically significant difference between the groups ($p = 0.003$) (Table 6).

No sub-analysis between different tooth types was performed since the results are presented only for moderate (PD 4–6 mm) and deep sites (PD ≥ 7 mm) without including furcation involved teeth.

Discussion

The present randomized clinical trial has investigated the clinical outcomes obtained with the subgingival application of a combination of sodium hypochlorite/amino acid and xHyA gels in conjunction with non-surgical periodontal therapy in untreated periodontitis patients. The results have shown that in patients diagnosed with stages II–III periodontitis, SRP combined with sodium hypochlorite/amino acid and xHyA gels resulted in statistically significantly higher clinical improvements evidenced through PD reduction, CAL gain, and decrease of BOP and PI values as compared to SRP alone.

An interesting observation of the study is related to PD and CAL changes between 3 and 6 months in moderate pockets. In particular, no statistically significant change was observed in the control group between the 3- and 6-month follow-ups, whereas in the test group, the change reached statistical significance. This observation appears to indicate that the test group demonstrated gradual improvements from month 3 to month 6, even though no additional treatment was performed. This finding may bear clinical relevance since it may suggest that the clinical improvements following the adjunctive sodium hypochlorite/amino acid and xHyA to SRP occur over a longer period of time (e.g., up to 6 months). Additionally, this observation may also suggest that a period of 3 months following nonsurgical periodontal therapy might be too early for making a final decision on the need for additional therapy (e.g., periodontal surgery). A similar pattern supporting the gradual improvement, was also observed for FMBOP and FMPI, where no statistically significant differences were observed between the groups at the 3-month follow-up, while it reached statistical significance at 6 months in favour of the test group.

This observation might be explained by the mode of action of xHA. In particular, the high molecular weight cross-linked HA that was used in this clinical trial can maintain its stability for 4 to 6 weeks which in turn, may serve as explanation for its prolonged activity [33].

When interpreting the clinical outcomes, it must be emphasized that the goal of non-surgical periodontal treatment is PD ≤ 4 mm with negative BOP [34]. The results of the current study have shown that the need for further treatment appears to be smaller in the test group, as demonstrated by the analysis of the change of number of moderate (4–6 mm) and deep pockets (≥ 7 mm) over time. In detail, in the control group, the total number of pockets with PD 4–6 mm decreased from 1518 to 803 with the corresponding values of 1803 and 234 in the test group. Similarly, the number of deep sites reduced from 277 to 35 in control and from 298 to 4 in test group.

As stated by Salvi et al., generally, a PD reduction of approximately 1–1.5 mm in moderate pockets (4–6 mm) and 2–2.5 mm in deep pockets (≥ 6 mm) can be expected [35] following mechanical debridement. This occurs concomitantly with CAL gain of approximately 0.5 mm in moderate pockets at baseline and 1.5 mm in deeper sites [35]. Any additional pocket reduction or CAL gain would, therefore, represent a true clinical benefit of the adjunctive materials used. This observation was also confirmed in the present study where in moderately deep sites, the mean PD change from baseline to 6 months measured 1.7 mm in the control group and 2.9 mm in test group, respectively, with the corresponding values of 3.7 mm and 5.8 mm, at deep sites (PD ≥ 7 mm). In moderately deep pockets, the mean CAL gain from baseline to 6 months measured 1.6 mm in the control group and 2.6 mm in test group, while in deep pockets, the corresponding values measured 3.2 mm and 5.3 mm, respectively.

When interpreting the results, one may ask the question to what extent each of the used adjunctive materials contributed to the additional improvements observed in

Table 6 Number of sites with shallow (1–3 mm), medium (4–6 mm) and deep (≥ 7 mm) pockets in test and control groups at different study timepoints

	1–3 mm			4–6 mm			≥ 7 mm		
	Control	Test	*P* value	Control	Test	*P* value	Control	Test	*P* value
Baseline	1916 (51.2%)	1603 (42.7%)	**0.05***	1518 (41.2%)	1803 (48.6%)	**0.041***	277 (7.6%)	298 (8.7%)	0.52
After 3 months	2938 (78.6%)	3284 (88.2%)	**0.013***	728 (20.3%)	402 (11.5%)	**0.018***	39 (1.1%)	12 (0.3%)	0.053
After 6 months	2859 (76.4%)	3398 (92.2%)	**0.006***	803 (22.6%)	234 (7.7%)	**< 0.001***	35 (1.0%)	4 (0.1%)	**0.003***

Data in bold represents statistically significant differences between test and control groups

the test group. In this respect, it is important to emphasize that the present study has used the combination of the two materials as a single concept, thus combining the effects of sodium hypochlorite/ amino acid gel to facilitate mechanical debridement and biofilm removal with the well-known wound-healing facilitating effects of xHyA. Based on previous findings from *in vitro* and animal experiments, it was hypothesized that the inherent effect of NaOCl to facilitate mechanical debridement and biofilm removal, may lend additional support to xHyA to express its wound healing improving properties [20, 23–25].

Despite the inherent positive effects of the used combination approach, it should be kept in mind that combining two materials and their use in conjunction with scaling and root planing also means a higher therapy effort in terms of time and costs. Additionally, it should be also emphasized that the present has only evaluated the outcomes in moderate (PD 4–6 mm) and deep sites (PD ≥ 7 mm) at teeth without furcation involvement. Obviously, further studies are warranted to evaluate the potential effect of this treatment approach in furcation involved teeth.

However, to the best of our knowledge, this is the first RCT evaluating the outcomes following the adjunctive application of sodium hypochlorite/amino acid gel and xHyA to scaling and root planing for untreated periodontal disease.

A recently published retrospective analysis of 29 clinical cases evaluated the adjunctive application of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid (high molecular) gels to SRP for treating residual periodontal pockets in patients diagnosed with periodontitis stages II–IV who were included into periodontal maintenance [30]. The authors reported an overall PD reduction exceeding 2 mm, associated with a similar CAL gain (2.02 mm). The results are comparable with the results obtained in this study. However, it must be emphasized that the study included compliant patients who already underwent nonsurgical periodontal treatment, as well as patients diagnosed with periodontitis stage IV, and therefore, direct comparisons are difficult. However, the same protocol has been evaluated in a very recent case series consisting of a total of twenty-one systemically healthy, non-smoking patients diagnosed with stage II-III periodontitis [31]. Compared to baseline, a statistically significant mean reduction of PD values was obtained after 3- and 6- months, amounting 2.6 ± 0.4 mm, and 2.9 ± 0.4 mm, respectively ($p < 0.001$), while mean CAL gain measured 2.3 ± 0.5 mm at 3- months, and 2.6 ± 0.5 mm at 6-months in comparison to baseline ($p < 0.001$). Mean reduction of BOP values amounted to 54.9 ± 16.9% at 3- months, and to 65.6 ± 16.4% at 6-months, respectively ($p < 0.001$). The number of moderate pockets (4–5 mm) reduced from 1808 at baseline to 274 at 6 months evaluation, and the number of deep (≥ 6 mm) pockets changed from 319 to 3, respectively [31]. These results compare well to those obtained in the present study, thus pointing to the potential clinical relevance of this novel clinical protocol.

Moreover, the adjunctive application of sodium hypochlorite/amino acid and hyaluronic acid gels to SRP has been tested separately in several clinical studies. On one hand, a recent clinical trial has evaluated the effect of the adjunctive application of sodium hypochlorite gel to SRP in residual periodontal pockets [9]. The findings revealed statistically significant PD reduction favouring the used of the sodium hypochlorite/amino acid gel, compared to a placebo ($p = 0.028$), as well as a statistically significant CAL gain at 6 months in the NaOCl-treated group, compared to the application of CHX gel ($p = 0.0026$).

One the other hand, the results of the studies on the adjunctive application of hyaluronic acid to non-surgical periodontal therapy are inconsistent. For instance, some of the studies found statistically significant improvements for the adjunctive application of hyaluronic acid to SRP in terms of PD and BOP reductions and CAL gain [27, 29], whereas in other studies adjunctive application of hyaluronic acid did not reach statistically significant differences in the investigated clinical parameters compared to SRP alone [36, 37].

Obviously, when interpreting the current results, certain the following limitations need to be mentioned: a) the study included a relatively small sample size and was of relatively short duration (i.e., 6 months), and b) only systemically healthy, non-smoking patients diagnosed with periodontitis stages II and III exhibiting adequate oral hygiene skills were included in the study.

Conclusion

Within their limits the present data indicate that: a) Both treatments resulted in statistically significant improvements in all evaluated clinical parameters, and b) The adjunctive subgingival application of sodium hypochlorite/amino acid gel and xHyA to SRP yielded statistically significantly higher improvements compared to SRP alone.

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Data availability All data underlying the results are available as part of the article and no additional source data is applicable.

Declarations

Competing interests The authors declare no competing interests.

Conflicts of Interest The authors declare no potential conflict of interest with respect to the authorship and/or publication of this article.

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PERIODONTOLOGY

Clinical Evaluation of a Novel Combination of Sodium Hypochlorite/Amino Acid and Cross-linked Hyaluronic Acid Adjunctive to Non-surgical Periodontal Treatment: A Case Series

Egle Ramanauskaite[a] / Vita Machiulskiene[b] / Urte Marija Dvyliene[c] / Meizi Eliezer[d] / Anton Sculean[e]

Purpose: The adjunctive subgingival application of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid gels (high molecular weight) has been recently proposed as a novel modality to enhance the outcomes of non-surgical periodontal therapy. The aim of this prospective case series was to evaluate the clinical outcomes obtained following the subgingival application of a combination of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid (high molecular) gels in conjunction with non-surgical periodontal therapy.

Material and Methods: Twenty-one systemically healthy, non-smoking patients diagnosed with stage II-III, grade A/B periodontitis underwent full-mouth subgingival debridement (SD) performed with ultrasonic and hand instruments. All sites with probing depths (PD) ≥ 4 mm were treated with additional repeated (i.e., 2-3 times) instillation of sodium hypochlorite/amino acid gel in the periodontal pockets prior to and during SRP. Following mechanical debridement, a mixture of natural and cross-linked hyaluronic acid (high molecular) gel was applied in the pockets. The primary outcome variable was PD reduction; changes in clinical attachment level (CAL) and bleeding on probing (BOP) were the secondary outcomes. The clinical parameters were assessed at baseline, 3 and 6 months after therapy.

Results: Compared to baseline, a statistically significant mean reduction of PD values was obtained after 3 and 6 months, amounting to 2.6 ± 0.4 mm, and 2.9 ± 0.4 mm, respectively ($p < 0.001$). Mean CAL gain measured 2.3 ± 0.5 mm at 3 months and 2.6 ± 0.5 mm at 6 months in comparison to baseline ($p < 0.001$). Mean reduction of BOP values was 54.9 ± 16.9 % at 3 months and 65.6 ± 16.4 % at 6 months ($p < 0.001$). The number of moderate pockets (4-5 mm) decreased from 1808 at baseline to 274 at the 6-month evaluation, and the number of deep (≥ 6 mm) pockets dropped from 319 to 3, respectively.

Conclusion: The combination of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid (high molecular) adjunctive to subgingival debridement may represent a valuable approach to improve the outcomes of non-surgical periodontal treatment.

Key words: cross-linked hyaluronic acid, non-surgical periodontal therapy, periodontitis, sodium hypochlorite/amino acid.

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Periodontitis is a chronic, progressive disease, characterised by expansion of the polymicrobial biofilm at the gingival margin, with the formation of an inflammatory infiltrate that contributes to destruction of connective-tissue attachment to the tooth, alveolar bone resorption and eventually even tooth loss.[1,2,27]

Dental plaque biofilm represents an acquired tissue of bacterial origin that maintains the health of gingival tissues and facilitates interactions between microorganisms and the host.[4,10] In periodontitis, a disruption of the normal function of the healthy subgingival plaque biofilm is observed, with concomitant disruption of its functional properties, leading to excessive, deregulated inflammation and tissue destruction.[4,13]

Elimination of the biofilm is a key element for the successful treatment of periodontitis. Although thorough subgingival debridement is a cornerstone of periodontal therapy, its effective-

ness may be limited by several factors (e.g., deep periodontal pockets, intrabony defects, furcation involvement, operator's manual skills, the patient's smoking status, etc.). Therefore, the adjunctive application of antimicrobial chemotherapeutic agents to eliminate or inactivate the periodontal pathogenic microflora at sites where mechanical instrumentation is cumbersome is highly clinically relevant.[22]

It has been suggested that adjunctive aids may enhance the outcomes of mechanical debridement.[22,24,28] Recently, the novel concept of 'Clean and Seal' – based on adjunctive use of two components, i.e., sodium hypochlorite/amino acid (Perisolv, Regedent; Zürich, Switzerland) and a mixture of natural and cross-linked hyaluronic acid (high molecular) (Hyadent BG, Regedent) gels along with mechanical instrumentation – was introduced as an option for non-surgical periodontal therapy.

In fact, preclinical studies have shown that sodium hypochlorite/amino acid gel acts antiseptically in particular against gram-negative species associated with periodontitis and is able to alter biofilm matrices.[3] Moreover, hyaluronic acid demonstrated bacteriostatic effects on bacterial strains associated with periodontitis and was proven to be beneficial in minimising bacterial contamination of surgical wounds.[11]

Regarding the 'Clean and Seal' concept, the cleaning effect is achieved by the activity of the sodium hypochlorite/amino acid gel. Laboratory experiments have demonstrated that sodium hypochlorite/amino acid gel has a softening effect on the extracellular matrix of the biofilm[14] and therefore, during treatment, both mechanical and chemical reactions act in concert to disrupt the biofilm and remove granulation tissue.[23] It is noteworthy that this chemomechanical method has no detrimental effect on sound dentin and/or root cementum. The high pH of the product affects calculus and has a softening effect, which makes the cleaning process easier to perform.[23]

The sealing effect is obtained by subsequent application of a mixture of natural and cross-linked hyaluronic acid gel (high molecular). Hyaluronic acid is a major constituent of the extracellular matrix of the skin, joints, eye, and many other tissues and organs.[18] Numerous in-vitro studies have provided evidence that hyaluronic acid stimulates blood clot formation,[26] induces angiogenesis[6] and enhances osteogenesis.[5,15,25] In addition, hyaluronic acid was found to play a key role in each phase of wound healing by stimulating cell migration, differentiation, and proliferation.[18]

However, at present, clinical data validating the clinical efficacy of the aforementioned treatment concept in patients with untreated periodontitis is lacking. Therefore, the aim of this prospective case series was to evaluate in patients with untreated periodontitis the clinical outcomes obtained with subgingival application of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid (high molecular) in conjunction with non-surgical periodontal therapy.

MATERIALS AND METHODS

Subject Selection

A total of 21 systemically healthy patients were recruited from new referrals to the Department of Dental and Oral Pathology, Lithuanian University of Health Sciences. The inclusion criteria were: a clinical diagnosis of stage II-III periodontitis,[20] at least one pocket in each quadrant with pocket depth (PD) ≥ 5 mm; radiographic evidence of bone loss (> 2 mm from cemento-enamel junction [CEJ]); a minimum of 20 teeth (wisdom teeth excluded); no removable prosthesis. The exclusion criteria were: patients already included in other clinical trials; smokers; periodontal treatment during the last 12 months; antibiotic treatment 6 months prior to the start of the trial; antibiotic prophylaxis required for dental treatment; ongoing medication that may affect the clinical features of periodontitis; pregnancy/lactation.

Furthermore, patients were included in the study if they exhibited an adequate level of oral hygiene evidenced by full-mouth plaque score (FMPS) <25%[19] and full-mouth bleeding score (FMBS) <25%.[16] Written informed consent was obtained from all patients. Ethical approval was obtained from Kaunas Regional Biomedical Research Ethics Committee (2018-BE-2-87).

Treatment

Baseline periodontal measurements were obtained two weeks prior to treatment, which was followed by professional supragingival tooth cleaning and individual oral hygiene instructions for all of the included patients. Oral hygiene instructions were reinforced at each follow-up visit, but no further treatment was provided.

Two weeks later, under local anesthesia, all patients underwent full-mouth SD performed with ultrasonic (Satelec/Acteon suprasson newtron ultrasonic scaler; Merignac, France) and hand instruments (LM SharpDiamond 1/2, 7/8, 11/12, 13/14 SD mini Gracey and Gracey curettes). Subsequently, all teeth were polished using a low-abrasive paste (Lunos Super Soft, RDA<5, Dürr Dental; Bietigheim-Bissingen, Germany). Per patient, the average time needed for the treatment was 3 h. All teeth with probing depths (PD) ≥ 4 mm were treated with sodium hypochlorite/amino acid gel (Perisolv, Regedent) injected into the periodontal pockets 60 s prior to and during SD (2-3 times) (Fig 1). No additional rinsing with saline was performed. Mechanical debridement was followed by the subsequent application of a mixture of natural and cross-linked hyaluronic acid (high molecular) gel (Hyadent BG, Regedent) in periodontal pockets measuring ≥ 4 mm (Fig 2).

Periodontal treatment was performed by an experienced periodontist (E.R.).

All patients were advised to follow their regular home oral-hygiene regimen and to refrain using antiseptic mouthwashes during the entire study period.

Clinical Assessments

The following clinical parameters were assessed using a Williams periodontal probe to the nearest mm (LM 51 ES, LM-Dental; Parainen, Finland) at all teeth at six sites per tooth (i.e., mesio-buccal [mb], mid-buccal [b], disto-buccal [db], mesio-oral [mo], mid-oral [o] and disto-oral [do]) at baseline, 3 and 6 months post-treatment:

- Bleeding on probing (BOP) assessed through visual inspection 20 s after probing using a dichotomous scale (present/absent)

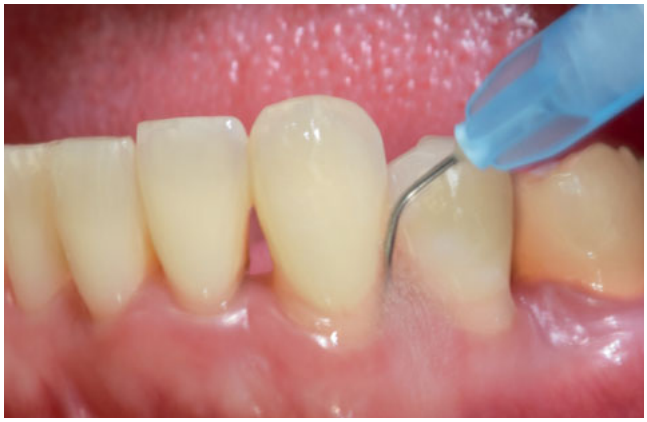

Fig 1 Application of sodium hypochlorite/amino acid gel to the periodontal pocket.

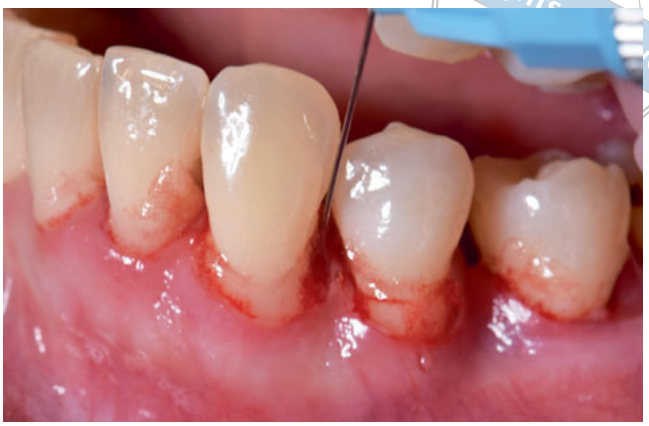

Fig 2 Application of a mixture of natural and cross-linked hyaluronic acid (high molecular) to the periodontal pocket.

- Probing depth (PD) measured in mm from the gingival margin to the bottom of the probed pocket
- Recession (REC) measured in mm from the gingival margin to the cemento-enamel junction or to the margin of a cervical restauration
- Clinical attachment level (CAL) calculated by adding PD and REC at each site

Clinical assessments were performed by a calibrated examiner (U.M.D.) who was not aware of the procedure to be performed. Before the beginning of the study, five patients not involved in the study, each diagnosed with periodontitis stages II–III,[20] were used to calibrate the examiner. The examiner was asked to evaluate PD, REC, CAL and BOP at 6 sites per tooth at 2 separate appointments, 48 h apart. Calibration was accepted if measurements at baseline and at 48 h were equal to the nearest mm at the >90% level.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 27 software (IBM; Armonk, NY, USA). The primary outcome variable was the reduction of PD. The Shapiro-Wilk test was performed to assess whether clinical periodontal measures followed a normal distribution. Statistical analysis was based on the Wilcoxon signed-rank test to assess pre- and post-treatment comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

This case series comprised a total of 21 healthy non-smoking patients, 15 females (71.4%) and 6 males (28.6%). The age of the included patients ranged from 33 to 75 years old, with a median age 50 years.

Descriptive statistics for PD, CAL, and BOP at baseline of the study are summarised in Table 1.

Compared to baseline, a statistically significant mean reduction of PD was obtained after 3 and 6 months, amounting to 2.6 ± 0.4 mm and 2.9 ± 0.4 mm, respectively ($p < 0.001$).

The difference in PD reduction between the 3- and 6-month follow-ups was 0.3 ± 0.3 mm, and was statistically significant ($p = 0.004$). Compared to baseline, mean CAL gain amounted to 2.3 ± 0.5 mm at 3 months, and 2.6 ± 0.5 mm at 6 months ($p < 0.001$). A statistically significant difference of CAL gain was measured between the 3- and 6-month follow-up visits (0.4 ± 0.4 mm, $p = 0.016$).

A statistically significant reduction of mean BOP values was noted after 3 and 6 months following treatment. In particular,

Table 1 Descriptive characteristics of sample population at the baseline

Patients (n)	21
Median age (range)	50 (33–75)
Gender, n (%) Males Females	 6 (28.6) 15 (71.4)
PD (mm) Mean ± SD	4.7 ± 0.2
CAL (mm) Mean ± SD	4.9 ± 0.5
BOP (%) Mean ± SD	83.2 ± 15.6

Table 2 Means (SD) of the differences (Δ) from baseline for probing depth (PD), clinical attachment level (CAL) and bleeding on probing (BOP)

	Month 3	Month 6	Δ 3 to 6 months
Δ PD (mm)	2.6 (0.4)	2.9 (0.4)	0.3 (0.4)
Δ CAL (mm)	2.3 (0.5)	2.6 (0.5)	0.4 (0.4)
Δ BOP (%)	54.9 (16.9)	65.6(16.4)	10.7 (11.9)
Statistically significant ($p < 0.001$).			

Table 3 Number of sites with shallow (1–3 mm), medium (4–5 mm) and deep (> 6 mm) pockets at different timepoints

	1–3 mm	4–5 mm	≥ 6 mm
Baseline	1603	1803	319
After 3 months	3224	414	9
After 6 months	3375	274	3

a mean reduction of BOP values compared to baseline reached 54.9 ± 16.9 % at 3 months and 65.6 ± 16.4 % at 6 months ($p < 0.001$). The BOP reduction between 3- and 6-month follow-ups amounted to 10.7 ± 11.9 % ($p < 0.001$).

Means (SD) of the differences vs baseline for PD, CAL and BOP are depicted in Table 2.

Furthermore, the frequency distribution of shallow (1-3 mm), medium (4-5 mm) and deep (> 6 mm) pockets at baseline, at 3 and at 6 months was analysed (Table 3). At baseline, study subjects exhibited 1803 sites with PD 4-5 mm, which decreased to 414 and 274 sites at the 3- and 6-month follow-ups, respectively. The number of sites ≥ 6 mm decreased from 319 at baseline to 9 at 3 months and to 3 at 6 months.

DISCUSSION

The present prospective case-series study investigated the clinical outcomes obtained with subgingival application of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid (high molecular) gels in conjunction with non-surgical periodontal therapy.

The findings suggest that the adjunctive application of a combination of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid (high molecular) gels to scaling and root planing in pockets exhibiting a PD ≥ 4 mm led to statistically significant improvements of the investigated clinical parameters. In particular, at 6 months after treatment, the mean PD reduction was 2.9 ± 0.4 mm ($p < 0.001$), while the mean CAL gain measured 2.6 ± 0.5 mm ($p < 0.001$). The mean BOP decreased from 83.2 ± 15.5% at baseline to 17.6 ± 11.5 % at the 6-month follow-up ($p < 0.001$). Interestingly, a statistically significant improvement in PD, CAL and BOP values was observed from the 3- to the 6-month follow-up, even though no further treatment was performed.

An important finding of the present study is the change in the total number of moderate (4-5 mm) and deep (≥ 6 mm) pockets. In particular, the total number of pockets of 4-5 mm was reduced from 1803 to 274 with the corresponding values of 319 and 3 in the deep-pocket (≥ 6 mm) category. As the ultimate goal of non-surgical periodontal therapy is to reduce/eliminate all sites > 4 mm, the 'Clean and Seal' technique seemed to be efficient in reducing further treatment need for the residual periodontal pockets.

Another important finding was the uneventful healing of soft tissues; none of the patients reported any adverse reactions or discomfort following therapy. These findings are important, since to the best of our knowledge, this is the first clinical study evaluating the effectiveness of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid (high molecular) gels in conjunction to SRP in patients with untreated periodontitis.

Justification for the adjunctive application of hyaluronic acid has been provided by several clinical studies.[7,12,21] Previous clinical data pointed to statistically significant reductions in PD[7,12,21] and BOP,[12,21] as well as CAL gains,[21] following adjunctive hyaluronic acid applications compared to subgingival debridement alone. In line with this, findings of one recent systematic review on non-surgical periodontal therapy pointed to a statistically significant reduction in PD (weighted mean difference (WMD): 0.36 mm; 95%CI: 0.54 to -0.19 mm; $p<0.0001$), BOP values (-15%; 95%CI: -22 to -8%; $p<0.0001$) and CAL gain (0.73 mm; 95% CI:

0.28 to 1.17 mm; p<0.0001) following adjunctive topical application of hyaluronic acid over SRP alone.[8]

Clinical effectiveness of the adjunctive use of sodium hypochlorite gel has been evaluated in several clinical studies reporting on non-surgical treatment of residual periodontal pockets (PD ≥5 mm),[17] non-surgical peri-implant mucositis[9] and peri-implantitis therapy.[23] In particular, while treating residual periodontal pockets, greater PD reduction in initially deep residual pockets (≥7 mm) was observed in the adjunctive sodium-hypochlorite gel group. Furthermore, following treatment, only one residual pocket of ≥ 7 mm was still detectable in a test group, whereas six compromised sites persisted in the control group.[17] Regarding non-surgical peri-mucositis therapy, the adjunctive application of sodium hypochlorite gel led to slightly better PD reduction compared to the control (i.e., mechanical debridement) – from 3.93 ± 1.09 mm to 3.04 ± 0.46 mm (p = 0.0001) and from 3.68 ± 0.85 mm to 3.07 ± 0.58 mm (p = 0.0001), respectively. However, no statistically significant difference was observed between the groups (p=0.53).[9] Similar results were observed when adding sodium hypochlorite gel adjunctively in non-surgical peri-implantitis therapy.[23] In particular, the reduction of BOP-positive sites in the test group changed from 0.97 (SD ± 0.12) to 0.38 (SD ± 0.46), and in the control group from 0.97 (SD ± 0.12) to 0.31 (SD ± 0.42), but there were no statistically significant differences between the study groups.

Despite the fact that no statistically significant improvements in PD and BOP could be obtained in the studies mentioned above,[9,17,23] the test groups always showed a tendency for greater clinical improvements than the controls (i.e., mechanical debridement), thus pointing to the beneficial effect of the adjunctive application of sodium hypochlorite.

When interpreting the results, it must be pointed out that the present case series only provides data from 21 consecutively treated patients without a control group (i.e., SD alone) and with a relatively short follow-up period (i.e., 3 and 6 months). However, despite these limitations, the excellent clinical outcomes coupled with the uneventful healing seem to suggest that this novel treatment concept may be of clinical relevance, thus warranting further investigations. Obviously, randomised controlled clinical trials are needed to validate this treatment concept for non-surgical periodontal therapy.

CONCLUSION

Within its limitations, the present case series has shown that a combination of sodium hypochlorite/amino acid and a mixture of natural and cross-linked hyaluronic acid (high molecular) adjunctive to subgingival mechanical debridement may represent a valuable approach to improve the outcomes of non-surgical periodontal treatment.

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Chloramine gel and hyaluronic acid gel enhance effect of non-surgical instrumentation in persistent residual periodontal pockets – preliminary report from a clinical case series

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Introduction

„SRP is not free of limitations, and its impact in some patients … or in specific sites my be not enough to achieve the desired results.“ (Herrera et al., 2020)

Objectives:

This retrospective study evaluated the effect of Clean & Seal concept as adjunctive therapy applied to persistent periodontal pockets at non-surgical subgingival instrumentation in patients who underwent supportive periodontal therapy (SPT) service.

Material and Methods:

- Patients from the SPT program at the Department of Periodontology who disclaimed history of at least one site with persistently increased probing depth > 5 mm with positive BoP at previous 2-3 SPT visits were enrolled.
- Clinical attachment level (CAL), periodontal probing depth (PPD), gingival recession (GR) and bleeding tendency (BoP) were assessed before intervention and at following SPT visits.
- Intervention included subgingival application of Chloramine gel (Perisolv; Regedent, Germany) for ≈ 30 sec (=„Clean“) followed by subgingival instrumentation with hand and/or power driven instruments for one to two cycles; the subgingival xHyA (Hyadent BG, Regedent Germany) application completed the treatment sequence and was repeated within following seven days (=„Seal“).
- Non-parametric statistics (Wilcoxon rank test) was performed after testing for normal distribution using Prism 9 software (GraphPad, La Jolla, CA, USA).

Results:

- Total of 29 patients 54.6 years of age (range: 39-75) presented with 111 defects and from 1 to 17 involved teeth each.
- 69% females and 31% males (n= 20 vs. n= 9) participated.
- Two patients were smokers, all 29 patients were normoglycemic.
- The total of 111 sites composed 99 sites without Furcation Involvement (FI), whereas 12 sites were located at either mesial or distal furcation wall (**Table 1, 2, 3**).
- The baseline PPD mean value was 7.19±1.89 mm and baseline CAL loss mean value 7.96±2.2 mm, calculated for all sites respectively (**Table 1**).
- The 6 month reevaluation revealed an overall mean PPD value of 5.16±1.81 mm and 5.95±1.8 mm for CAL, respectively. None additional recession from baseline to 6 months follow-up occurred; the mean gain in CAL was estimated with 2.02 mm and corresponded to the mean of 2.04 mm in PPD reduction.
- The PPD reduction and CAL gain were statistically significant (P<.0001, respectively) at 6 month reevaluation (**Table 1**; **Fig. 1 a+b**).
- The BoP frequency was reduced by 60% (**Fig. 2**).
- **Table 2 and 3** disclose the separately calculated values for 99 sites without furcation involvement vs. 12 sites from the furcation area. **Figure 3** shows level of statistical significance.
- Meanwhile several patients completed the 12 month follow-up SPT visit and sites were examined by periapical x-rays. The x-rays displayed clinically relevant gain in newly organized mineralized tissue within former infrabony defect extensions (**Figure 4 a-d**).

Table 1:

		Pre	Post	Gain
cAL	Mean (SD)	7.96 (±2.2)	5.95 (±1.8)	+2.02mm
	Median	7	6	
	Min	2	2	
	Max	9	13	
PPD	Mean (SD)	7.19 (±1.89)	5.16 (±1.81)	-2.04mm
	Median	6	5	
	Min	4	2	
	Max	15	12	
BOP		97.6%	40.1%	

Figure 2:

Figure 1 a+b:

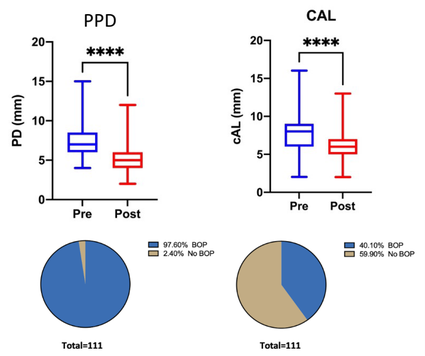


Table 2 and 3:

Furcation involved (n=12)		Pre	Post	Gain
cAL	Mean (SD)	9.08 (±2.88)	7.58 (±1.73)	+1.50mm
	Median	9	8	
	Min	6	4	
	Max	16	9	
PPD	Mean (SD)	8.25 (±2.59)	5.83 (±1.75)	-2.42mm
	Median	8	5.5	
	Min	6	3	
	Max	15	9	

No furcation involved (n=99)		Pre	Post	Gain
cAL	Mean (SD)	7.93 (±2.03)	5.89 (±1.87)	+2.04mm
	Median	7	6	
	Min	5	2	
	Max	13	13	
PPD	Mean (SD)	6.96 (±1.68)	5.15 (±1.86)	-1.81mm
	Median	6	5	
	Min	4	2	
	Max	12	12	

Figure 3:

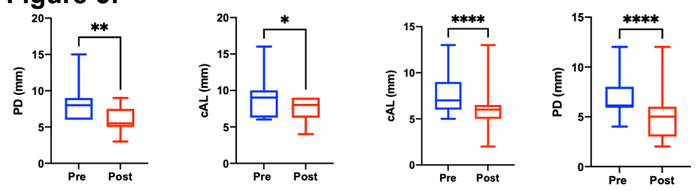
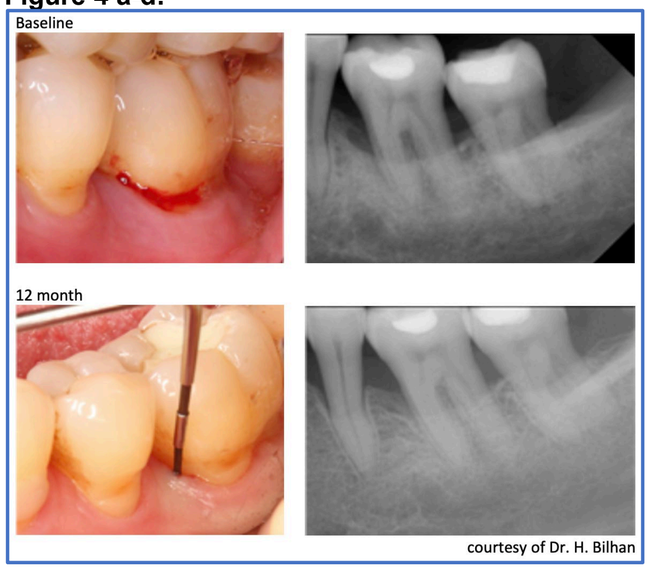


Figure 4 a-d:



Conclusion:

These preliminary data indicated that the adjunctive use of chloramine gel together with repeated xHyA application sufficiently enhanced the outcome of subgingival instrumentation in previously persistent sites as expressed by clinical parameters which were partially corroborated by x-rays. Higher number of cases and RCT studies required to confirm these observations.

materials

MDPI

Article

Adjunctive Application of Hyaluronic Acid in Combination with a Sodium Hypochlorite Gel for Non-Surgical Treatment of Residual Pockets Reduces the Need for Periodontal Surgery—Retrospective Analysis of a Clinical Case Series

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Abstract: The comprehensive treatment of periodontitis stage 2 to 4 aims at the resolution of periodontal inflammation and "pocket closure", which implies a residual probing depth of ≤4 mm and a negative BoP. However, supportive periodontal therapy (SPT) regularly leaves behind persistent periodontal pockets with 5 or more mm in residual PPD and sites that often re-colonize and re-infect. Various adjunctive options for subgingival instrumentation have been proposed to enhance the antimicrobial effects to better control the re-infection of these residual sites. The locally applied adjuncts, based on their anti-inflammatory effect, are sodium hypochlorite antiseptic cleaning gel and cross-linked hyaluronic acid (xHyA). Both recently moved into the focus of clinical research on non-surgical and surgical therapy for periodontitis. The surgical use of xHyA indicates regenerative potential, supporting periodontal regeneration. This case series retrospectively analyzes the clinical benefits of the consecutive flapless application of sodium-hypochlorite-based cleaning gel and xHyA at the SPT to achieve pocket closure, thereby reducing the need for periodontal surgery. In 29 patients, 111 sites received the treatment sequence. At 6-month re-evaluation, an overall PPD reduction exceeding 2 mm was achieved, associated with a similar CAL gain (2.02 mm); the bleeding tendency (BoP) was reduced by >60%. Pocket closure occurred in almost 25% of all the sites. Within their limits, the present data suggest that the proposed combined adjunctive treatment of residual active periodontal sites yielded significant improvement in the clinical parameters. Further studies in RCT format are required to confirm these observations.

Keywords: hyaluronic acid; sodium hypochlorite; periodontitis; non-surgical periodontal therapy

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1. Introduction

Non-surgical periodontal treatment (NSPT) results in improved probing depth, clinical attachment level, and bleeding tendency [1]. The purpose of NSPT is the resolution of periodontal inflammation and a reduction in pocket-probing depth (PPD) to 4 mm or less, resulting in pocket closure. However, residual or recurring pockets exhibiting PPD values ≥4 mm are regularly found at re-evaluation. Residual periodontal pockets facilitate the accumulation of biofilm, leading to dysbiosis within the re-colonized subgingival habitat and, thus, to persistent inflammation [2,3]. Moreover, long-term data confirm the association between residual PPD and increased risk of tooth loss [4]. Therefore, as recommended in the European Federation of Periodontology (EFP) guidelines, continuous supportive periodontal therapy (SPT) accompanied by repeated instrumentation is imperative for sustained periodontal stability [5].

In an effort to improve the outcome of non-surgical instrumentation, a variety of adjunct treatment modalities are used. In addition to systemic antibiotics, a plethora of locally administered adjunctives seek to minimize both PPD and bleeding tendency, thereby facilitating the closure of the periodontal pocket.

Most of these adjunctive treatments are based upon the antimicrobial effects delivered by either photodynamic therapy (PDT) or the use of local antibiotic chemotherapy, preferably applied as a device with sustained release kinetics [6–10]. Furthermore, gelatin chips sustainably releasing chlorhexidine have been described [11–13]. Addressing the limitations of subgingival instrumentation on pocket-closure frequency, a recent systematic review and meta-analysis evaluated the additional benefit of locally applied adjunctive therapies. Even though the authors found effects of statistical significance, the magnitude of these benefits was deduced to be rather irrelevant to clinical success in terms of pocket closure [14]. Furthermore, the microbiological analysis of samples retrieved from persistent deep pockets before and after repeated local metronidazole application revealed high counts of periodontal pathogens [9].

By contrast, a novel amino-acid-buffered sodium hypochlorite cleaning gel exhibiting antimicrobial potential was significantly effective in improving the outcome of non-surgical therapy and, thus, significantly reduced counts of Gram-negative pathogens in an artificial biofilm model [15,16].

Another strategy to improve periodontal parameters is the local administration of regenerative biologics. In an attempt to harness its well-documented regenerative properties, a recent multi-center randomized controlled trial investigated the effect of enamel-matrix derivatives (EMDs) as an adjunct to the NSPT of patients situated in SPT [17]. The authors were able to show significantly greater pocket closure for sites treated with adjunctive EMD, demonstrating biologics-based regenerative technologies as promising supplements for non-surgical therapy.

Furthermore, a review with a meta-analysis showed that the adjuvant non-surgical administration of hyaluronic acid (HA) resulted in an improvement in both clinical attachment and probing depth [18]. Currently, however, there is a lack in protocols for adjuncts to NSPT combining both antimicrobial and regenerative properties. In this retrospective case series, we propose a novel two-step approach consisting of an amino-acid-buffered sodium hypochlorite cleaning gel to assist in the decontamination of the root surface, followed by the concomitant application of a cross-linked hyaluronic acid gel (xHyA) to facilitate healing and, thus, pocket closure. We report the retrospective analysis of 6-month clinical follow-up data from patients who qualified for this therapy.

2. Materials and Methods

The local ethics committee at the Witten/Herdecke University approved this retrospective evaluation of a clinical case series (S-203/2021). All the analyzed cases had been diagnosed with stage 2 to 4 periodontitis previously and had already undergone comprehensive periodontal therapy, as proposed by the EFP guidelines [5,19]. Four calibrated specialists and residents at the Department of Periodontology of Witten/Herdecke University were responsible for all treatment steps. Calibration of investigators was evaluated by analysis of variance (ANOVA), followed by Tukey's post hoc analysis for multiple comparisons ($p > 0.59$ for all investigators). The decision to administer systemic antibiotics strictly complied with the EFP guidelines, following completion of initial subgingival instrumentation.

2.1. Inclusion Criteria

The proposed treatment applied to sites that exhibited persistent deep pocket depths after patients had undergone consecutive SPT re-evaluations at least twice. Sites ascribed to the treatment by protocol had never been subjected to any surgical intervention, even though patients may have received periodontal surgery at other sites. Specifically, persistent and recurrent periodontal pockets displaying $\geq$5 mm in PPD with positive BoP were included. The number of sites per patient assigned to the therapy was unrestricted. There

was no limit to the localization of residual or recurrent pockets, and single- and multi-rooted teeth were included. In teeth with high PPD associated with furcation involvement of more than Class 1, only the change in vertical component of the defect was analyzed for this report.

2.2. Treatment Sequence

Four calibrated operators treated all patients; the operators agreed upon the treatment protocol before the first application. Following supragingival mechanical instrumentation, each site received subgingivally administrated sodium hypochlorite cleaning gel (Perisolv; Regedent AG, Zürich, Switzerland) for 30 to 45 s to support chemical disinfection and improve the scaling outcome. Subgingival instrumentation was carried out with Gracey curettes (Deppeler, American Dental Systems, Munich, Germany). The sodium hypochlorite cleaning-gel application was repeated until the instrumentation was considered sufficient (Figure 1). Sufficient instrumentation was attained when root surfaces exhibited smooth surfaces upon probing with an explorer probe (ODU 11/12 DH2, Deppeler, Rolle, Switzerland). Subsequently, 0.3 mL of the cross-linked hyaluronic acid gel (xHyA; hyaDENT BG, Regedent AG, Zürich, Switzerland) was applied into the subgingival pocket in a flapless manner until plenished. Patients were instructed to uphold daily mechanical biofilm control by means of interdental brushes and a toothbrush. Measures for oral hygiene were not adjusted in the operated area. Neither systemic antibiotics nor antiseptics for rinsing were prescribed by protocol. Within the next 7 days, a repeated subgingival xHyA application (0.3 mL) was conducted combined with the oral hygiene control. The first re-evaluation took place 5–6 months after treatment and the subsequent SPT interval was set to 3 months for a 12-month period. At the 12-month re-evaluation, a periapical radiograph taken with the parallel technique was obtained to verify the crestal bone level.

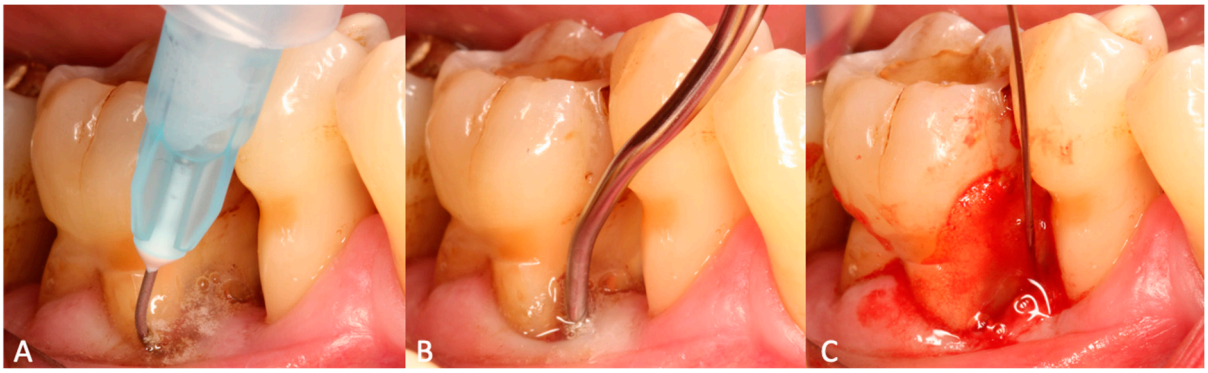

Figure 1. Visualization of the applied treatment protocol. (**A**) Application of chloramine gel to the pocket for 30–45 s. (**B**) Scaling and root planning is performed. Chloramine gel may be applied repeatedly until non-surgical treatment is deemed sufficient. (**C**) Cross-linked hyaluronic acid (xHya) is applied to the pocket until plenished.

2.3. Statistical Analysis

For all obtained datasets, a descriptive data analysis was performed. Further statistical analyses included the Shapiro–Wilk, Kolmogorov–Smirnov, and D'Agostino–Pearson tests to assess data distribution. CAL gain and PPD reduction (pre–post) were both calculated by Wilcoxon signed-rank test, respectively. p-values of ≤ 0.05 were considered significant.

3. Results

This retrospective analysis included 29 patients with 111 treated teeth/sites, ranging from 1 to 17 per patient. The mean age was 54.6 years, and 69% were female (20:9; 69% vs. 31%). All patients were normo-glycemic and 7% (n = 2) were smokers. Table 1 discloses the

demographics, habits, and health condition of the participants. All of them participated in the SPT program offered by the Department of Periodontology.

Table 1. Patient demographics and mean clinical parameters before (pre) and after (post = 6 months) the treatment. CAL = clinical attachment level, PPD = probing pocket depth, BOP = bleeding on probing, * = Wilcoxon signed-rank test.

Patients (Sites)			**29 (111)**	
Mean Age (Range)			**54.6 (39–75)**	
Sex				
– **Male (%)**			**9 (31%)**	
– **Female (%)**			**20 (69%)**	
Smokers (%)			**2 (7%)**	
Diabetes (%)			**0**	
		Pre	**Post**	**CAL gain/PPD Reduction**
CAL	Mean (SD)	7.96 (±2.2)	5.95 (±1.8)	+2.02 mm ($p < 0.0001$) *
	Median	7	6	
	Min	2	2	
	Max	9	13	
PPD	Mean (SD)	7.19 (±1.89)	5.16 (±1.81)	−2.04 mm ($p < 0.0001$) *
	Median	6	5	
	Min	4	2	
	Max	15	12	
BOP		97.6%	40.1%	

The mean PPD at baseline was 7.19 (±1.89) mm, and the CAL loss was 7.96 (±2.2) mm; 97.6% of all sites presented with positive BoP. Consecutive six-month re-evaluation revealed an overall PPD reduction of 2.04 mm and a clinical attachment level gain of 2.02 mm, indicating that no further progression in gingival recession occurred. The BoP frequency decreased to 40.1%. Stratified by furcation involvement (12 teeth), the mean CAL gain was 1.5 mm ($p = 0.0195$), whereas the treatment of single-rooted teeth resulted in a 2.04 mm ($p < 0.001$) CAL gain (Tables 2 and 3, Figure 2). Both measurements yielded statistically significant differences compared to the baseline values. In terms of pocket closure, 25 out of 99 (25.25%) sites in the single-rooted teeth exhibited pocket closure, with a PPD < 4 mm and a negative BoP.

Table 2. Descriptive statistics of PPD and CAL development in furcation-involved sites after combined chloramine and xHya treatment. Pre = baseline, Post = 6 months post treatment, * = Wilcoxon signed-rank test.

Furcation Involved (n = 12)		**Pre**	**Post**	**CAL Gain/PPD Reduction**
CAL	Mean (SD)	9.08 (±2.88)	7.58 (±1.73)	+1.50 mm ($p = 0.0195$) *
	Median	9	8	
	Min	6	4	
	Max	16	9	
PPD	Mean (SD)	8.25 (±2.59)	5.833 (±1.75)	−2.42 mm ($p = 0.002$) *
	Median	8	5.5	
	Min	6	3	
	Max	15	9	

Table 3. Descriptive statistics of PPD and CAL development in sites without furcation involvement after combined chloramine and xHya treatment. Pre = baseline, Post = 6 months post treatment, * = Wilcoxon signed-rank test.

No Furcation Involved (n = 99)		Pre	Post	CAL Gain/PPD Reduction
CAL	Mean (SD)	7.93 (±2.03)	5.89 (±1.87)	+2.04 mm ($p < 0.0001$) *
	Median	7	6	
	Min	5	2	
	Max	13	13	
PPD	Mean (SD)	6.96 (±1.68)	5.15 (±1.86)	−1.81 mm ($p < 0.0001$) *
	Median	6	5	
	Min	4	2	
	Max	12	12	

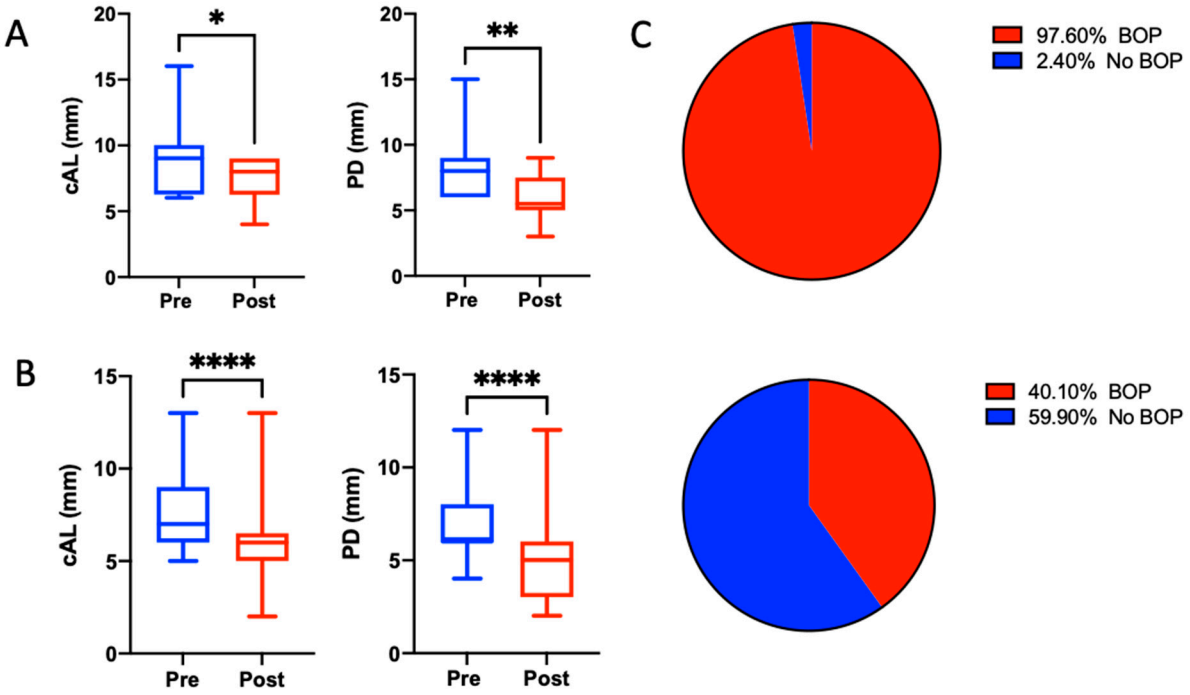

Figure 2. Boxplots for clinical parameters before and after the treatment sequence of non-furcation-involved (**A**) and furcation-involved (**B**) sites. Whiskers represent minimum and maximum values. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (**C**) Amount of sites exhibiting bleeding on probing before (upper) and after (lower) the treatment.

4. Discussion

This retrospective case series shows that the combination of an antiseptic adjunctive cleaning gel and xHyA applied subgingivally for the treatment of persistently deep periodontal pockets at SPT visit yielded clinically relevant improvements in PPD reduction, CAL gain, and BoP frequency. The follow-up of the reported cases revealed statistically significant improvement in all three of these parameters. The overall CAL gain exceeded 2 mm on average in sites previously classified as non-responding and persistent. Although a minor number of treated sites exhibited complete pocket closure after three to six months, the two-component flapless adjunctive treatment considerably reduced the need for periodontal surgery. Sites ascribed to surgical step3 therapy according to the EFP guidelines clinically improved to such an extent that the periodontal surgery became redundant. To the best of our knowledge, this is the first report of the combined use of antiseptic and

biologic approaches in flapless periodontal treatment. As each site received both adjunctive materials administered at one visit, we must emphasize that a discussion of the individual contributions to the results appeared unnecessary.

Recent in vitro, pre-clinical, and clinical studies investigated either the sodium hypochlorite cleaning gel or the xHyA application in a separate manner. The antimicrobial effects of the sodium hypochlorite cleaning gel became evident [16,20]. Cell-based experiments also disclosed the high level of cytocompatibility of its compounds [20,21]. However, the benefits of adjunctive sodium hypochlorite cleaning gel for NSPT remain controversial. Sodium hypochlorite gel failed to affect the clinical outcome of ultrasonic or manual subgingival instrumentation in SPT treatment. Nevertheless, its use was associated with significantly reduced recolonization of the sites by *T. denticola* and *T. forsythia* [22]. By contrast, the adjunctive benefit of sodium hypochlorite gel formulation for minimally invasive non-surgical therapy (MINST) was positively evaluated by a recent RCT [15]. The authors compared the outcome of step-2 therapy after delivering it to untreated stage-3 and -4 periodontitis patients in both study arms. Moreover, in an RCT study from a Scandinavian research group, diabetic foot ulcers resolved significantly quicker under treatment with this cleaning gel formulation than those in the control group [23].

Hyaluronic acid (HA) is a glycosaminoglycan heteropolysaccharide and, in its native form, it is both a light-molecular-weight (LMWHA) and a high-molecular-weight long polymer (HMWHA) [24]. HA is an important natural component of the extracellular matrix and is almost ubiquitously present in mammalian tissues, including the periodontium [25]. Several studies confirmed bacteriostatic [26,27], fungostatic [28], anti-inflammatory [29], anti-edematous [30], osteoinductive [29,31–33], and pro-angiogenic [34] properties of HA. In animal studies on skin wounds, HA promoted enhanced connective-tissue elasticity and healing, improved re-epithelialization, and appeared to increase microvascular density [34,35]. HA sufficiently improved wound healing in extraoral wounds, skin ulcers, and intraoral injuries [36–38].

The potential of xHyA to promote periodontal regeneration became a subject in a recent series of histological evaluations in dogs' mandibles. The histomorphometric assessments revealed that xHyA-treated intraosseous and furcation sites formed significantly greater areas of new cementum and periodontal ligament fibers on previously exposed root surfaces. Similar observations were made from the same treatment sequence applied in gingival recessions [39–41].

The clinical results mediated by xHyA indicated a substantial benefit, which was corroborated by both a recent RCT study and a case series [42,43]. Beyond the positive effects of xHyA unfolded in the surgical context, its adjunctive use in NSPT yielded inconsistent outcomes in clinical studies [44–46].

In our retrospective analysis, we found a significant probing-depth reduction accompanied by a significant gain in clinical attachment (Figure 2). Moreover, the needlessness of root conditioning and drying the wound area increased the ease of handling and delivered strong arguments in favor of xHyA as an adjunct to flapless subgingival instrumentation, as well as accounting for its hygroscopic/wound-stabilizing and regenerative properties. In addition, compliance with the second visit scheduled for repeated xHyA application was high in all the patients. With respect to the proposed protocol, the sodium hypochlorite cleaning-gel application may offer further advantages to NSPT by means of improving the mechanical biofilm removal, thus enhancing the effects of the xHyA. Therefore, we consider the proposed protocol highly beneficial for NSPT. However, the presented results require further confirmation by randomized controlled clinical trials, which may also account for the exposure time and application frequency of the hypochlorite gel.

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

Data Availability Statement: The data presented in this study are available on request from the corresponding authors. The data are not publicly available because they were derived from patients.

Conflicts of Interest: The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Clinical and microbiological effects of a single application of sodium hypochlorite gel during subgingival re-instrumentation: a triple-blind randomized placebo-controlled clinical trial

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Abstract

Objectives The aim of this study is to assess the clinical and microbiological effects of a single subgingival administration of sodium hypochlorite gel (NaOCl) and compare it with 1% chlorhexidine (CHX) gel and a placebo gel following mechanical re-instrumentation during supportive periodontal therapy (SPT).

Materials and methods Sixty-two patients who had been treated for stage III–IV periodontitis and enrolled in SPT were included in the study based on following criteria: (1) active periodontal therapy completed at least 6 months before enrollment in the study, (2) presence of at least 4 non-adjacent sites with probing pocket depths (PPDs) ≥ 4 mm with bleeding on probing (BOP), or presence of 5–8 mm PPDs with or without BOP. All sites presenting PPD ≥ 4 mm and BOP at baseline and 3-, 6-, and 9-month follow-up timepoints were subgingivally re-instrumented with ultrasounds. Selected patients were randomly assigned into three groups and treated additionally with a single subgingival administration of NaOCl gel (group A); 1% CHX gel (group B); and placebo gel (group C). Main outcome variable was pocket closure at 12 months. Secondary outcome variables were changes in mean PPD, BOP, and clinical attachment level (CAL) along with changes in the numbers of the following five keystone bacterial pathogens: *Aggregatibacter actinomycetemcomitans* (*A.a.*), *Porphyromonas gingivalis* (*P.g.*), *Prevotella intermedia* (*P.i.*), *Tannerella forsythia* (*T.f.*), and *Treponema denticola* (*T.d.*).

Results At 12 months, pocket closure was obtained in 77.5% in the NaOCl treated sites. The reduction in PPD was higher with CHX than with NaOCl, although a statistically significant adjunctive effect for NaOCl ($P = 0.028$) was only observed in comparison with placebo only. Mean CAL improved in all groups and at all timepoints, compared to the baseline ($P < 0.05$). However, after 6 months, CAL gain was statistically significantly higher in the NaOCl treated group than following application of CHX ($P = 0.0026$).

Conclusion In SPT patients, a single adjunctive use of a NaOCl gel may provide benefits in controlling inflammation and residual pockets.

Trial registration ISRCTN Registry of Clinical Trials (ISRCTN11387188).

Clinical relevance A baseline single application of NaOCl gel in conjunction with mechanical debridement may achieve substantial pocket closure in patients enrolled in SPT; treatment time, cost, and applicability considerations should be taken into account when selecting this therapy.

Keywords Periodontal maintenance · Subgingival re-instrumentaion · Sodium hypochlorite · Probing pocket debridement

Introduction

Substantial evidence has shown that periodontitis is triggered and maintained by dysbiosis of the periodontal pathogenic biofilm and subsequent destructive inflammatory response. Consequently, treatment of periodontitis

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always focuses in all phases on the mechanical removal or destruction of the supra- and subgingival biofilm [1–5].

Subgingival re-instrumentation during supportive periodontal therapy (SPT) has been shown to result in additional clinical improvements in only about 50% of affected sites, as evidenced by a reduction in probing pocket depths and bleeding on probing, while the rest of affected sites may show further disease progression [6–8].

The goals of SPT are to minimize or prevent recurrence of the disease and/or arrest its progression to maintain long-term periodontal health and chewing comfort [9–11]. Substantial evidence indicates that SPT plays a key role in arresting periodontal disease prognosis and increases tooth survival [12–16]. It is recommended that SPT starts once the endpoint of active periodontal therapy (APT) is reached (i.e., PPD ≤ 4 mm, absence of BOP of 4 mm sites) [17–20].

Mechanical disruption of the biofilm is an effective approach and is still considered as the "gold standard"; it is sometimes limited by the inadequate access and visibility to the operative sites [21, 22]. Air polishing devices have been proposed as a more effective alternative for biofilm removal at sites difficult to access with hand curettes or machined driven instruments, since the stream of abrasive particles can remove biofilm residues which may remain after conventional instrumentation [23]. Recent data provide evidence suggesting that air polishing devices may represent a valuable modality for biofilm removal during SPT [24]. However, the rationale of performing repeated subgingival scaling at 3-month intervals for patients with persistent disease has been questioned [25], thus pointing to the need, in specific clinical scenarios, of using adjunctive antimicrobials having as main rationale the antimicrobial effect at sites that are inaccessible to mechanical therapy thus increasing the possibility of reaching and destroying remaining pathogens [26]. Local delivery systems containing antibiotic or antiseptic drugs allow therapeutic agents to target diseased sites with minimal systemic effects [27]. Compared to use of SRP only, the combined use of several local anti-infective agents and scaling and root planning (SRP) seems to provide additional benefits in PPD reduction and clinical attachment level (CAL) gain [28]. Within the last decade, topical slow-release antimicrobials, such as chlorhexidine, doxycycline, minocycline, and metronidazole, have been used subgingivally in conjunction with mechanical instrumentation during SPT [29–33]. Substantial evidence indicates that adding a chemotherapeutic agent to conventional SPT has an adjunctive effect in interrupting further periodontal disease progression, as observed in persistent or recurrent periodontitis after local use of doxycycline [6, 31, 34]. The adjunctive application of an antimicrobial agent may be also useful for patients with contraindications of surgery and patients with extreme sensitivity after active periodontal treatment [32].

A recent study [35] has evaluated the potential benefit of an enamel matrix derivative (EMD) as an adjunct to re-instrumentation of residual pockets during the step 3 of periodontal therapy [20]. The frequency of pocket closure in the test group was statistically significantly higher than in the control group at 6 months and was maintained up to 12 months.

Very recently, the use of sodium hypochlorite (NaOCl) has been also suggested as a possible alternative to improve the outcomes of subgingival SRP. This is mainly due to its broad antimicrobial activity, fast bactericidal action, and non-toxicity at application concentration [36, 37]. Histologically, subgingival application of (NaOCl) provides chemolysis of the soft tissue wall of the periodontal pocket with minimal effect on the adjacent tissues. Hence, its use in the maintenance phase of periodontal therapy has been recommended [38].

Antimicrobials which are currently use adjunctively in subgingival re-instrumentation during SPT (i.e., mainly antibiotics and CHX) have been associated with potential risks of antimicrobial resistance [37, 39]. For instance, the oral cavity has been highlighted as potential reservoir for antimicrobial resistance genes in numerous publications from recent years [40, 41]. NaOCl could be an interesting alternative because its mechanism of action is rather non-selective (oxidative burst) as opposed to antibiotics or CHX [42]. Thus, development of resistances toward NaOCl seems less likely as toward antibiotics or CHX.

Recently, a novel formulation of NaOCl gel (Perisolv, RLS Global AB, Gothenburg, Sweden) buffered with leucine, lysine, and glutamic acid was used as an adjunct to subgingival instrumentation [43] and re-instrumentation [44] for the treatment of peri-implant mucositis [45] and peri-implantitis [46]. The active ingredients in the gel create chloramines, which have a strong antimicrobial effect and can penetrate the biofilm [44], thus making an alternative approach to improve the outcomes of ultrasonic re-instrumentation (USI) procedures [47, 48]. An in vitro study indicated that the NaOCl gel had antimicrobial activity against Gram-negative species associated with periodontitis, although it failed to eliminate a multi-species biofilm [40].

The phase of therapy at which other topical slow-release antimicrobials are most beneficial remains unclear. However, these formulations appear to be most beneficial when used during SPT at non-responding or recurrent chronic inflammation sites [49].

Accordingly, to the best of our knowledge, at present, only one study has addressed the issue of topical NaOCl gel in re-instrumentation of persistent pockets during SPT [44]. However, in that study, the treatment consisted of repeated topical applications of the novel hypochlorite gel in conjunction with short-time ultrasonic debridement. As other studies have indicated, the existing data on the potential clinical relevance of local application of NaOCl gel used in conjunction with subgingival mechanical instrumentation remains limited [43].

Therefore, the aim of this triple-blinded randomized placebo-controlled clinical study was to compare the clinical and microbiological effects between the adjunctive subgingival administration of NaOCl gel and chlorhexidine and a placebo gel with subgingival re-instrumentation and air polishing during the first 12 months of SPT.

Material and methods

Study design

This study was conducted as a triple-blinded randomized placebo-controlled clinical trial of 12 months with a parallel design of three independent groups by a 1:1:1 allocation ratio. The study was approved by the Research Ethics Committee of the Victor Babes University of Medicine and Pharmacy Timisoara (approval no.1/21.01.2018). The study was conducted according to the principles outlined in the Declaration of Helsinki on human medical experimentation. All participants provided written informed consent, giving permission for the dental procedures and sampling of biological material. The study was conducted between January 2018 and September 2019. The study was registered in the ISRCTN Registry of Clinical Trials (ISRCTN11387188) and followed the guidelines described in the CONSORT 2010 statement on clinical trials.

Study population

Out of 85 randomly selected and screened patients, 62 patients agreed to participate in the study. The participants were randomly assigned to one of the three study groups: groups A, B, and C. Not more than 50% of the patients were smokers. With respect to smoking, the patients were distributed in three groups: smokers (> 10 cigarettes/day regularly), former smokers, and non-smokers [50].

Patients that were included in the study had completed APT and received SPT for a minimum of 6 months of documented SPT, until the desired number of participants was attained. APT was performed in a private practice in Timisoara, Romania, whereas SPT was performed in a private practice and in the Department of Periodontology, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania. A flowchart of the study according to CONSORT is provided in Fig. 1.

The inclusion criteria were as follows:

(a) Patients aged 20–80 years
(b) Patients enrolled in SPT after at least 6 months following APT for periodontitis stages III–IV
(c) **A**t least four non-adjacent sites with PPDs ≥ 4 mm with BOP or PPDs > 5 mm, but not deeper than 8 mm, with or without BOP, needing retreatment ("reference sites") [6]
(d) Neither furcation involvement, nor third molars or severely malpositioned teeth
(e) Vital teeth or teeth with "lege-artis" root canal treatment
(f) Full mouth bleeding score (FMBS) ≤ 20%
(g) Full mouth plaque score (FMPS) ≤ 20%
(h) Mobility degree ≤ 2 [53]
(i) Patients treated (no surgical/surgical if indicated) in the same private practice where the study was conducted.
(j) Patients willing to provide written informed consent and willing to complete the 12-month study follow-up.

Exclusion criteria:

(a) Known allergies or adverse reactions to hypochlorite
(b) Clinically relevant psychological disorders
(c) Alcohol abuse
(d) HIV infection
(e) Self-reported diabetes mellitus
(f) Use of local or systemic administration of antibiotics during the last 3 months
(g) Pregnancy and breast feeding
(h) Heavy smokers. If progression of periodontal destruction was observed or if adverse reactions to the test product were reported, the participant was excluded from the study. Progression of periodontal destruction was defined as attachment loss > 2 mm or an increase in PPD > 2 mm between subsequent evaluations [6, 26].

Clinical examination

The clinical examination team included an examiner (specialist in periodontology), a randomizer, and an operator (specialist in periodontology) with at least 4 years of clinical experience. The intra-examiner calibration for reliability testing resulted in $\kappa = 0.92$ for repeated measurements of PPD and CAL in two quadrants of five patients, other than the patients recruited for the study. Periodontal diagnosis was made according to the new classification system for periodontal and peri-implant diseases and conditions (2018) [51]. Each patient's medical history was updated.

All clinical measurements (i.e., at baseline, at 3-, 6-, 9-, and 12-months) were performed by the same investigator (SS). Additionally, FMPS and FMBS were calculated [52]. PPD, gingival recessions (REC), and clinical attachment levels (CAL) were measured at six sites per tooth using a manual periodontal probe (PCP-UNC15, Hu-Friedy, Chicago, IL, USA). Measurements were recorded to the nearest millimeter. Mobility was recorded according to the Miller classification [53]. Periodontal parameters were recorded in the periodontal chart (http://www.periodontalchart-online.com/uk/), saved in "pdf" format, printed, and included the observation file of each patient.

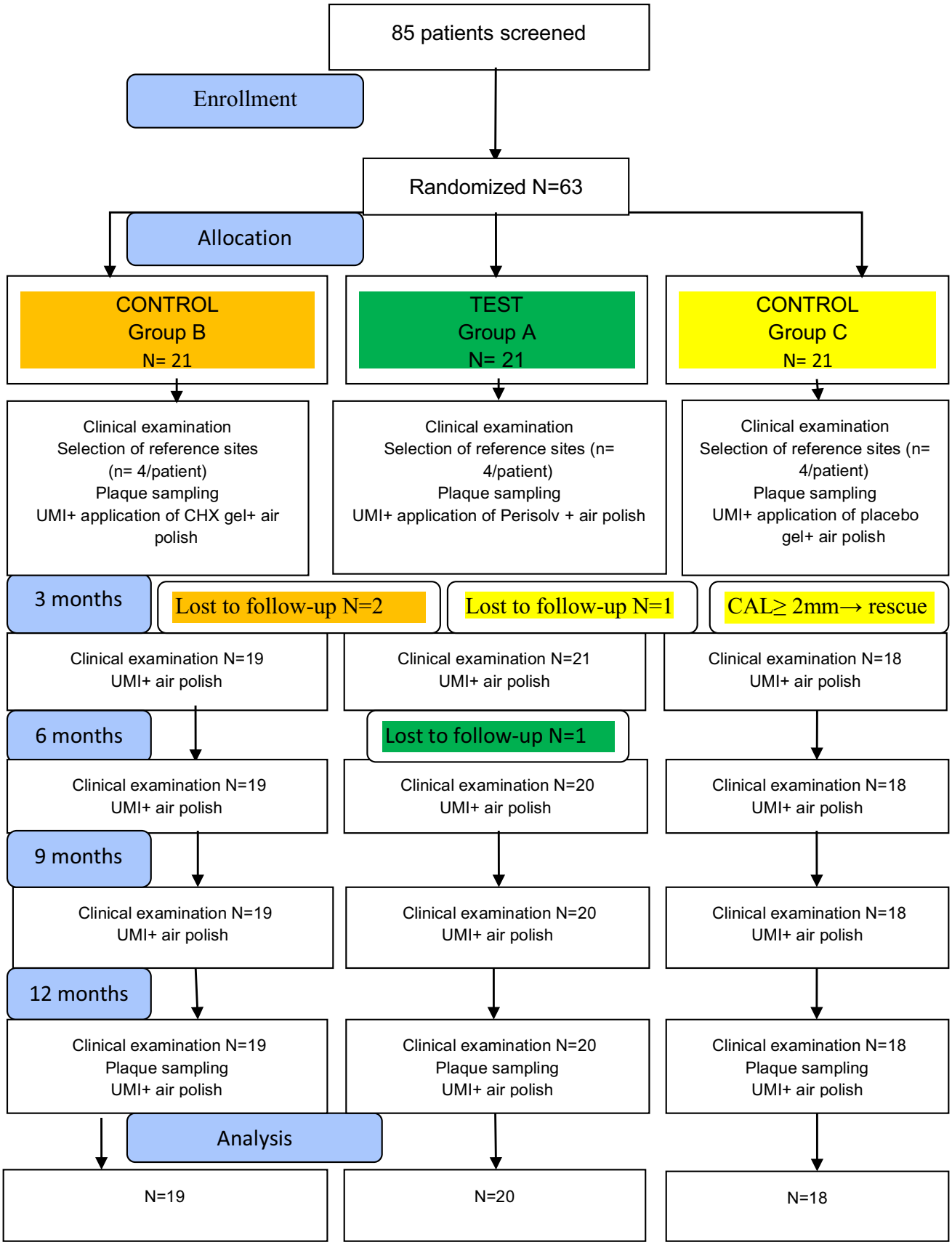

Fig. 1 CONSORT flow chart of patient enrolment and follow-up examination

Microbiological examination

To detect the selected bacteria, *Aggregatibacter actinomycetemcomitans* (*A.a.*), *Porphyromonas gingivalis* (*P.g.*), *Prevotella intermedia* (*P.i.*), *Tannerella forsythia* (*T.f.*), and *Treponema denticola* (*T.d.*), a molecular genetic analysis was performed. The semi-quantitative analysis of bacteria was assessed using the commercial kit, micro-IDent® plus (Hain Lifescience GmbH, Nehren, Germany), which is based on DNA STRIP technology. The microbiological samples were collected by the treating clinician (VR) from the teeth with the deepest PPD recorded at the initial evaluation. The microbiological samples at the 12-month re-evaluation time point were harvested exactly from the same sites. Subgingival plaque was collected for microbiological examination as follows. First, the site was isolated with cotton rolls. After removing the supragingival plaque and the debris with a sterile cotton gauze, the gingival surface was dried. The plaque samples were collected by inserting one sterile paper point ISO #30 in each one of the four reference sites and allowing them 30 s in situ for saturation [54]. The paper points were pooled immediately into sterile-sealed Eppendorf tubes and sent for polymerase chain reaction (PCR). The PCR testing was conducted in the laboratories of the Department of Biochemistry, Victor Babeş University of Medicine and Pharmacy. The cones were removed after 15 min of vortex mixing at room temperature, and the eluates were clarified by centrifugation for 5 min at 3000 × g at 23 °C. The samples were stored for one day at – 20 °C, and then at – 80 °C until the microbiological analysis was performed (not more than 30 days later).

Randomization and therapy assignment

Randomization was achieved using a number generator (www.randomizer.org) by a randomizer who was independent of the operator or evaluator. The randomizer ensured blinding by using a placebo gel similar in aspect and consistency to the test gel. Moreover, neither the patients, operator, nor clinical examiner knew the groups the patients were assigned. The computerized randomization assigned the patients to one of the three groups by an allocation ratio of 1:1:1. The randomizer performed the assignment to interventions, while a dental assistant performed the documentation. An allocation table containing the names of the patients was created and used to assign patient treatment numbers, as indicated by the randomization process. Each patient was given a sealed opaque envelope containing the treatment number.

SPT procedures

The operator (VA) performed the supragingival debridement (EMS Piezon® Master, EMS, Nyon, Switzerland) and air polishing (standard air-flow nozzle, AIRFLOW® PLUS powder (EMS, Nyon, Switzerland) at all sites. The reference sites and all sites presenting PPD ≥ 4 mm at baseline and 3-, 6-, and 9-month follow-up timepoints were re-instrumented with USI using fine subgingival inserts (PS (Perio Slim) EMS, Nyon, Switzerland) in the context of regular SPT. The NaOCl gel, chlorhexidine gel, or placebo gel was not reapplied at the 3-, 6-, and 9-month timepoints.

The investigated antimicrobial product (Perisolv®, Regedent AG, Zürich, Switzerland) consisted of two components contained in two separate interconnectable syringes: 0.95% sodium hypochlorite solution and transparent gel (the activating vehicle), containing amino acids (glutamic acid, leucine, lysine), carboxymethylcellulose, and ultrapure water. The two components were mixed before use to generate chloramines [44]. The chlorhexidine product (Clorhexamed® 1% gel, GSK, Germany) and placebo treatment consisted of gels with similar aspect and consistency as the test product, packaged in transparent syringes and identical with the syringe for the test product.

In group A, the reference sites were additionally treated as follows. According to the manufacturer's instructions, Perisolv® was applied by interconnecting the two syringes and mixing the liquids by alternately pushing the plungers. It was mixed until the liquid became homogeneous (10–15 cycles) and was then pushed into the transparent syringe. A blunt applicator was applied to this syringe and was inserted into the pocket mesially, lingually, distally, and buccally to cover the full circumference of the teeth and reach the bottom of the pocket. Perisolv® gel was left in situ for 30 s after application, followed by USI. After 15 min, Perisolv® was applied again, and teeth were re-instrumented subgingivally after 30 s using USI. Air polishing was used on all teeth to destroy the biofilm. In groups B and C, the reference sites were additionally treated with the chlorhexidine gel and placebo gel. The gels were applied in the same manner as in group A. For USI, no time limitations were set, and instrumentation was performed without local anesthesia until the treating clinician felt comfortable with the debrided root surfaces.

During the first periodontal re-evaluation, the investigator asked patients if any allergy or adverse reactions occurred after the treatment procedure, or if they had used medication that might interfere with the inclusion criteria. If necessary, the individual's oral hygiene was reinforced.

The participants were instructed to avoid using any other local or systemic antimicrobials. Oral hygiene instructions that were given to all participants during the initial periodontal therapy (i.e., use of rotary toothbrushing, dental floss, interdental brushes, pulsated water jet) were repeated and reinforced during each visit of the SPT. The timeline of the study is presented in Fig. 2.

Data analysis

The statistical analyses were performed using the software R version 4.0.0 (R Development Core Team, R Foundation

for Statistical Computing, Vienna, Austria) [55]. Statistical analysis was conducted intra- and inter-groups. The main outcome variable was pocket closure at the 12-month timepoint. Mean PPD changes, BOP, mean CAL changes, and the changes in the frequency detection scores of the five selected bacterial species were regarded as secondary outcomes. The sample size calculation was based on earlier reports on periodontal re-instrumentation [35, 56]. A minimal required sample size of 16 patients per group was required to achieve 80% power for detecting a statistically significant mean difference of 1 mm in the reduction of PPD between groups, assuming a common standard deviation of 0.8 mm and given significance level, $\alpha = 0.05$. The Pitman asymptotic relative efficiency correction was applied in the sample size computation to account for the use of nonparametric comparison tests. At least 18 patients were enrolled in each of the 3 groups to account for possible attrition. For each of the quantitative variable, PPD, REC, and CAL, a patient mean value was computed per timepoint, which was further used in the statistical analyses. For quantitative data, intergroup comparisons were made using the Kruskal–Wallis tests with Mann–Whitney post-hoc tests. Differences within each group from baseline to later timepoints (3, 6, 9, and 12 months) were analyzed using Wilcoxon signed-rank tests. Chi-squared tests or Fisher's exact tests, as appropriate, were used for comparisons between groups in the case of qualitative data. Statistical significance was set at $P < 0.05$.

Regarding the microbiological status, changes in the detection frequency scores of major keystone bacteria were assessed. Results were recorded and classified into one of the following categories: 0 = nondetectable, 1 = detectable $< 10^4$ (10^3 for *A.a*), 2 = 10^4–10^5 (10^3–10^4 for *A.a*), 3 = 10^5–10^6 (10^4–10^5 for *A.a*), and 4 ≥ 10^7 (10^6 for *A.a*) [54]. Intra-group comparisons of detection scores of pathogen species between the baseline and 12-month re-evaluation timepoints were performed using Wilcoxon signed rank test. The Kruskal–Wallis test was used for inter-group comparisons of detection scores for each timepoint.

Results

No side or adverse effects related to any of the treatment procedures occurred in any of the patients. Table 1 presents the characteristics of the patients at baseline. Test and control groups showed no statistically significant differences regarding sex, smoking, age, FMPS, FMBS, and PPD at baseline. The intragroup distribution was well pondered. The PPD of the sites ranged from 4 to 7 mm at baseline. The mean PPD at baseline was 4.56 ± 0.46 mm for the Perisolv® group, 4.48 ± 0.36 mm for the chlorhexidine group, and 4.57 ± 0.46 mm for the placebo group (Table 2). Additionally, 83.75% of Perisolv® treated sites, 94.74% of chlorhexidine treated sites, and 95.83% of placebo sites were identified as BOP-positive after probing at baseline (Table 3).

Out of 85 individuals that were screened, 63 patients met the inclusion criteria, gave written informed consent to participate, and were included in the study. Due to attrition, 57 patients were available for examination after 12 months. During the study, two participants showed disease progression; therefore, they were excluded from the study to undergo standard therapy. Figure 1 presents the study flow chart according to the CONSORT guidelines.

Tooth types (incisors/canines/premolars/molars) of reference teeth were distributed among groups as follows: 15/9/26/30 for Perisolv®, 7/11/27/31 for Chlorhexidine, and 20/15/18/19 for placebo. A total of 228 reference sites

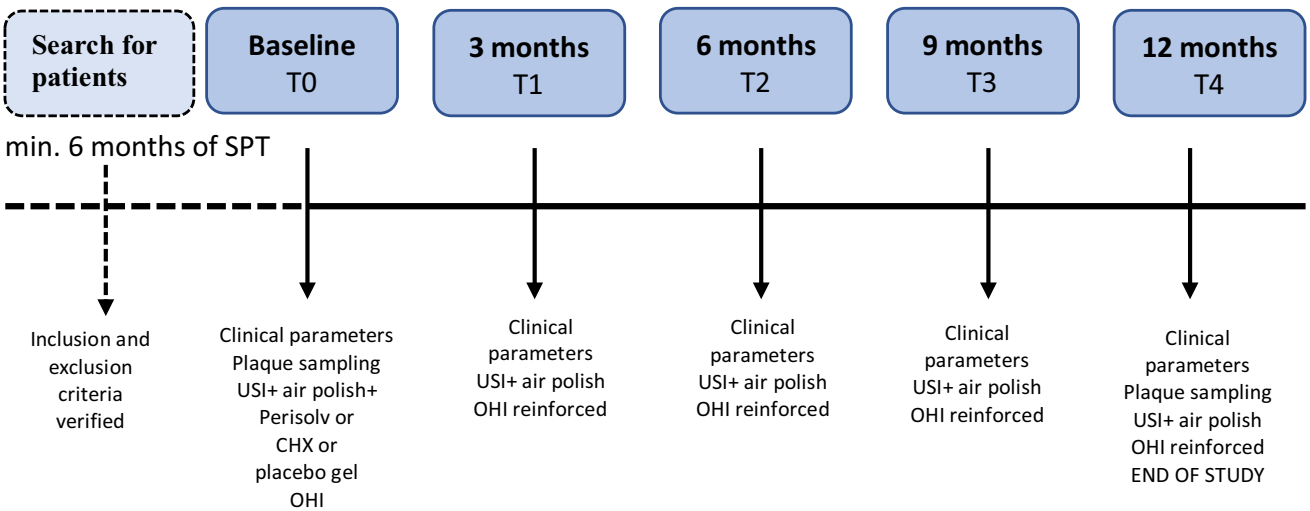

Fig. 2 Timeline of the study

Table 1 Characteristics of study participants at baseline

Parameter	Perisolv ($n=20$)	CHX ($n=19$)	Placebo ($n=18$)	p value
Age (years, mean ± SD)	44.60 ± 9.86	48.68 ± 11.63	50.61 ± 9.31	0.155[a]
Sex = female (n, %)	10 (50%)	8 (42.11%)	12 (66.67%)	0.313[b]
Smoker (n, %)	3 (15%)	3 (15.79%)	3 (16.67%)	1[c]
FMPS	15.10 ± 6.45	16.16 ± 6.11	16.33 ± 5.65	0.869[a]
FMBS	20.50 ± 4.32	20.16 ± 4.13	21.89 ± 2.11	0.608[a]
PPD = 4 mm (n, %)	46 (57.50%)	48 (63.16%)	39 (54.17%)	
PPD = 5 mm (n, %)	26 (23.50%)	22 (28.95%)	26 (36.11%)	
PPD = 6 mm (n, %)	5 (6.25%)	6 (7.89%)	6 (8.33%)	
PPD = 7 mm (n, %)	3 (3.75%)	0 (0%)	1 (1.39%)	

[a]Kruskal-Wallis test

[b]Chi-squared test

[c]Fisher's exact test

Table 2 Mean probing pocket depth (PPD) ± standard deviation (mm) at baseline and 3-, 6-, 9-, and 12-month timepoints in the treatment and control groups and p values of Kruskal–Wallis tests for intergroup comparisons

	PERISOLV	CHX	placebo	p-value
Baseline	4.56 ± 0.46	4.48 ± 0.36	4.57 ± 0.46	0.669
3 months	3.59 ± 0.42	3.66 ± 0.52	3.89 ± 0.64	0.127
Difference to baseline	0.98 ± 0.31	0.79 ± 0.36	0.68 ± 0.73	0.065
6 months	3.58 ± 0.35	3.76 ± 0.53	3.79 ± 0.72	0.343
Difference to baseline	0.99 ± 0.31	0.68 ± 0.45	0.78 ± 0.71	0.069
9 months	3.65 ± 0.43	3.71 ± 0.65	3.82 ± 0.58	0.524
Difference to baseline	0.91 ± 0.42	0.74 ± 0.58	0.75 ± 0.56	0.310
12 months	3.75 ± 0.47	3.84 ± 0.61	3.82 ± 0.57	0.934
Difference to baseline	0.81 ± 0.38	0.61 ± 0.52	0.75 ± 0.58	0.356

Table 3 Proportion of sites with BOP and p values of chi-squared tests for intergroup comparison

	PERISOLV	CHX	placebo	p-value
Baseline	67/80 (83.75%)	72/76 (94.74%)	69/72 (95.83%)	0.013
3 months	12/80 (15.00%)	15/76 (19.74%)	20/72 (27.78%)	0.147
6 months	18/80 (22.50%)	25/76 (32.89%)	20/72 (27.78%)	0.349
9 months	18/80 (22.50%)	22/76 (28.95%)	17/72 (23.61%)	0.615
12 months	10/80 (12.50%)	22/76 (28.95%)	23/72 (31.94%)	0.010

were treated. The four reference teeth were in different quadrants in 24 patients, and each reference site belonged to one

Table 4 Proportion of sites with pocket closure and p-values of chi-square tests for intergroup comparisons

	PERISOLV	CHX	placebo	p value
Baseline	0/80	0/76	0/72	–
3 months	64/80 (80.00%)	53/76 (69.74%)	46/72 (63.89%)	0.082
6 months	61/80 (76.25%)	51/76 (67.10%)	47/72 (65.28%)	0.281
9 months	61/80 (76.25%)	48/76 (63.16%)	46/72 (63.89%)	0.144
1 months	62/80 (77.50%)	48/76 (6 3.16%)	43/72 (59.72%)	0.044

reference tooth. The other 33 patients had a maximum of two reference teeth on the same quadrant (at least three teeth apart from each other), while the other two reference teeth were situated in the remaining three quadrants.

The primary outcome variable, pocket closure (Table 4), defined as the transition of sites with PPD > 5 mm or 4 mm with BOP to non-bleeding sites with PPD ≤ 4 mm, was attained in 77.5% of Perisolv® sites after 12 months. The reduction was higher in the CHX group than in the sodium hypochlorite gel group. However, a significant adjunctive effect of Perisolv® ($P=0.028$) was observed, when compared with the placebo group only at the 12-month timepoint. Therefore, the hypothesis tested could be confirmed only for one arm.

Periodontal re-instrumentation caused clinical improvements in both control and test groups, showing reductions in mean PPD value at test and control sites between baseline and 3-month follow-up timepoint. The results were maintained at subsequent re-evaluations (Table 2). However, these improvements, as well as differences between groups, were not statistically significant at any time point. Marginally, statistically significant differences were observed at the 3- and 6-month timepoints, favoring Perisolv® over

CHX and placebo. After 12 months of maintenance therapy, the mean PPD value of the study sites was reduced by 0.81 ± 0.38 mm in the test group, by 0.61 ± 0.52 mm in the CHX group, and by 0.75 ± 0.58 mm in the placebo group.

The analysis of BOP changes at test and control sites (Table 3) shows that the proportion of BOP sites in the Perisolv® group was significantly lower than in the CHX and placebo groups at baseline and at the 12-month timepoint. No difference in BOP incidence was recorded at 3-, 6-, and 9-month timepoints among study groups. The intra-group analysis showed an important decrease in the number of sites with BOP at the 3-month timepoint, followed by a stabilization tendency in all groups.

No statistically significant differences were identified in terms of REC changes among the study groups at any timepoint (Table 5). The intra-group analysis showed a statistically significant increase at 3-, 6-, and 9-month timepoint (Wilcoxon test, $P < 0.05$) from 0.29 ± 0.43 and 0.30 ± 0.57 to 0.40 ± 0.44 and 0.51 ± 0.67 for Perisolv and CHX group, respectively.

Although no statistically significant differences in terms of CAL changes were found among the groups at any timepoint (Table 6), an improvement occurred in all three groups compared to baseline (Wilcoxon tests, $P < 0.005$). Differences were observed among the groups when comparing the values from baseline with those from the 6-month timepoint (Kruskal–Wallis test, $P = 0.010$). Mann–Whitney post-hoc tests revealed that these differences were due to the more important 6-month CAL gain in the Perisolv® group than in the CHX group ($P = 0.0026$).

The intra-group analysis reveals a statistically significant decrease in detection scores from baseline to 12 months for *P.g.* (Perisolv®, CHX, and placebo group with *P* values of 0.015, 0.004, 0.002, respectively), *P.i.* (placebo group, $P = 0.049$), *T.f.* (Perisolv®, CHX, and placebo group, *P* value of 0.004, 0.003, and 0.010, respectively), and *T.d.* (Perisolv® and placebo groups with *P* value of 0.005 and 0.040, respectively). The inter-group analysis showed no statistically significant differences in the detection scores for *A.a.*, *P.g.*, *P.i.*, *T.f.*, and *T.d.* among the three groups, either at baseline or after 12 months (Table 7). In all three groups, pathogen detection scores either decreased over time or remained constant, with very few exceptions.

Table 5 Mean gingival recession (REC) ± standard deviation (mm) at baseline, 3, 6, 9, and 12 months in the treatment and control groups and p-values of Kruskal–Wallis tests for intergroup comparisons

	PERISOLV	CHX	placebo	*p*
Baseline	0.29 ± 0.43	0.30 ± 0.57	0.47 ± 0.69	0.635
3 months	0.43 ± 0.45	0.46 ± 0.66	0.58 ± 0.72	0.875
Difference to baseline	0.14 ± 0.19	0.16 ± 0.28	0.11 ± 0.23	0.656
6 months	0.40 ± 0.44	0.57 ± 0.67	0.61 ± 0.70	0.787
Difference to baseline	0.11 ± 0.21	0.26 ± 0.36	0.14 ± 0.25	0.299
9 months	0.36 ± 0.36	0.55 ± 0.69	0.61 ± 0.71	0.731
Difference to baseline	0.08 ± 0.28	0.25 ± 0.39	0.14 ± 0.26	0.496
12 months	0.40 ± 0.44	0.51 ± 0.67	0.65 ± 0.71	0.683
Difference to baseline	0.11 ± 0.15	0.21 ± 0.35	0.18 ± 0.32	0.781

Table 6 Mean clinical attachment level (CAL) ± standard deviation (mm) at baseline and 3-, 6-, 9-, and 12-month timepoints in the treatment and control groups and *p* values of Kruskal–Wallis tests for intergroup comparisons

	PERISOLV	CHX	placebo	*p*-value
Baseline	4.85 ± 0.70	4.75 ± 0.61	5.04 ± 0.82	0.531
3 months	4.01 ± 0.68	4.12 ± 0.65	4.47 ± 0.83	0.161
Difference to baseline	0.84 ± 0.37	0.63 ± 0.36	0.57 ± 0.60	0.078
6 months	3.98 ± 0.60	4.33 ± 0.64	4.40 ± 0.96	0.191
Difference to baseline	0.88 ± 0.35	0.42 ± 0.37	0.64 ± 0.62	0.010
9 months	4.01 ± 0.68	4.26 ± 0.70	4.43 ± 0.78	0.276
Difference to baseline	0.84 ± 0.46	0.49 ± 0.43	0.61 ± 0.46	0.062
12 months	4.15 ± 0.73	4.36 ± 0.69	4.47 ± 0.78	0.460
Difference to baseline	0.70 ± 0.40	0.39 ± 0.38	0.57 ± 0.50	0.095

Discussion

This study sought to evaluate the benefit of a single subgingival application of a low concentration hypochlorite/amino acid gel associated with subgingival USI and air polish in residual pockets ≥ 4 mm with positive BOP or residual pockets > 5 mm over a year of SPT.

The rationale for supplementary efforts aiming at improving periodontal maintenance and early intervention during SPT is confirmed by earlier observations that the current standard, based on repeated mechanical re-instrumentation of sites ≥ 4 mm and positive BOP, patient motivation, and oral hygiene instructions, is unable to control inflammation in more than 50% of sites. Although mechanical treatment substantially decreases the counts of subgingival microorganisms, it does not necessarily eliminate all periodontal pathogens [57].

The recently published S3-level clinical guideline for the treatment of periodontitis [20] tackles decision-making for retreatment after step 2 therapy (initial non-surgical phase). Based on the findings from a systematic review [58], it is recommended to re-instrument residual pockets with a PPD of 5 mm by a non-surgical approach. Residual pockets of ≥ 6 mm should be reduced by periodontal surgery to reach the endpoint of active therapy (PPD ≤ 4 mm, without BOP).

Table 7 Detection frequency scores for *A.a*, *P.g*, *P.i*, *T.f*, *T.d* at baseline and 12-month timepoint

Species	Timepoint	Detection score	PERISOLV	CHX	placebo	*p*-value**
A.a	Baseline	0	15 (75%)	14 (73.68%)	16 (88.88%)	0.408
		1	1 (5%)	1 (5.26%)	–	
		2	–	–	1 (5.56%)	
		3	1 (5%)	2 (10.53%)	1 (5.56%)	
		4	3 (15%)	2 (10.53%)	–	
	12 months	0	17 (85%)	16 (84.21%)	18 (100%)	0.218
		1	2 (10%)	1 (5.26%)	–	
		2	–	1 (5.26%)	–	
		3	1 (5%)	–	–	
		4	–	1 (5.26%)	–	
	p value*		0.098	0.181	0.371	
P.g	Baseline	0	6 (30%)	3 (15.79%)	1 (5.56%)	0.935
		1	1 (5%)	3 (15.79%)	1 (5.56%)	
		2	1 (5%)	1 (5.26%)	4 (22.22%)	
		3	3 (15%)	5 (26.32%)	6 (33.33%)	
		4	9 (45%)	7 (36.84%)	6 (33.33%)	
	12 months	0	11 (55%)	12 (63.16%)	9 (50%)	0.529
		1	1 (5%)	3 (15.79%)	–	
		2	2 (10%)	1 (5.26%)	2 (11.11%)	
		3	4 (20%)	–	3 (16.67%)	
		4	2 (10%)	3 (15.79%)	4 (22.22%)	
	p value*		**0.015**	**0.004**	**0.002**	
P.i	Baseline	0	5 (25%)	8 (42.10%)	6 (33.33%)	0.529
		1	4 (20%)	2 (10.53%)	1 (5.56%)	
		2	5 (25%)	6 (31.58%)	6 (33.33%)	
		3	6 (30%)	3 (15.79%)	5 (27.78%)	
		4	–	–	–	
	12 months	0	9 (45%)	12 (63.16%)	11 (61.11%)	0.354
		1	4 (20%)	3 (15.79%)	–	
		2	2 (10%)	4 (21.05%)	3 (16.67%)	
		3	5 (25%)	–	4 (22.22%)	
		4	–	–	–	
	p value*		0.121	0.095	**0.049**	
T.f	Baseline	0	–	–	–	0.325
		1	1 (5%)	1 (5.26%)	–	
		2	2 (10%)	2 (10.53%)	1 (5.56%)	
		3	4 (20%)	4 (21.05%)	11 (61.11%)	
		4	13 (65%)	12 (63.16%)	6 (33.33%)	
	12 months	0	8 (40%)	9 (47.37%)	6 (33.33%)	0.877
		1	1 (5%)	1 (5.26%)	1 (5.56%)	
		2	-	1 (5.26%)	1 (5.56%)	
		3	5 (25%)	2 (10.53%)	5 (27.78%)	
		4	6 (30%)	6 (31.58%)	5 (27.78%)	
	p value*		**0.004**	**0.003**	**0.010**	

Table 7 (continued)

Species	Timepoint	Detection score	PERISOLV	CHX	placebo	*p*-value**
T.d	Baseline	0	3 (15%)	4 (21.05%)	2 (11.11%)	0.121
		1	–	4 (21.05%)	7 (38.89%)	
		2	11 (55%)	9 (47.37%)	6 (33.33%)	
		3	6 (30%)	2 (10.53%)	3 (16.67%)	
		4	–	–	–	
	12 months	0	9 (45%)	8 (42.10%)	6 (33.33%)	0.860
		1	3 (15%)	5 (26.32%)	5 (27.78%)	
		2	4 (20%)	5 (26.32%)	6 (27.78%)	
		3	4 (20%)	1 (5.26%)	1 (5.56%)	
		4	–	–	–	
	p value*		**0.005**	0.078	**0.040**	

Data presented as frequencies (%)

* Corresponding to Wilcoxon tests for intra-group comparison of pathogen detection scores between successive timepoints

** Corresponding to Kruskal–Wallis tests for inter-group comparisons of pathogen detection scores for each timepoint

However, in the present study, a reduced number of sites with PD = 7 mm (3 in the test and one in the placebo group) were re-instrumented.

In a clinical trial from 1998 on chronic periodontitis [7], the authors noted that the average number of bleeding pockets per patient doubled over 5 years of SPT. PPD of 5 mm seemed to represent a risk factor for tooth loss, whereas residual PPD ≥ 6 mm represented an incomplete periodontal treatment outcome requiring further therapy [8]. The most relevant parameters used to assess the capacity of maintaining periodontal health and making supportive treatment useful are the percentage of sites with BOP and prevalence of residual pockets > 4 mm [59, 60]. These two parameters are easily affected by therapy.

Concurrently, the influence residual inflammation evidenced by BOP on tooth loss was addressed in many studies [60, 61]. Thus, the absence of BOP and PPD ≤ 4 mm (closed pockets) as clinical endpoints of treatment success is justified [17, 18]. According to Chapple et al. [19], periodontal stability is defined by a successful treatment resulting in minimal BOP (< 10% of sites) and PPD < 4 mm. For other authors [62], the reduction of PPD on a physiological level of up to 3 mm, which is the clinical pocket closure, remains the most important end parameter for clinically applicable success estimation after periodontal treatment.

Previous studies have assessed the effect of various adjunctive topical antimicrobial products in enhancing the outcomes of subgingival re-instrumentation of residual pockets during SPT [6, 30–32]. A recent study about the benefit of enamel matrix derivative (EMD) as an adjunct to re-instrumentation of residual pockets [35] was conducted according to the recently published S3-level clinical guideline for the treatment of periodontitis [20]. In that study, the authors explored the benefits of EMD as an adjunct to re-instrumentation of residual deep pockets with a PPD of 5–8 mm. The primary outcome was the change in mean PPD after 6 months. A statistically significant additional benefit of 0.79 ± 1.3 mm was observed in the test group and could be maintained until 12 months (0.85 ± 1.1 mm). In the present study, an additional benefit of 0.99 ± 0.31 mm was attained after 6 months for the test group and was maintained at the 12-month timepoint (0.81 ± 0.38 mm), although it was not statistically significant.

Regarding the change of residual deep sites to sites with shallow probing depth (PPD ≤ 4 mm), the frequency of conversion amounted to 76% at the 6-month timepoint and 80% at the 12-month timepoint for the test sites, compared to 46% and 45% for the control sites. In the present study, the frequency of PPD reduction was 76.25% at the 6-month timepoint and 77.50% at the 12-month timepoint for the test group and 63.89% at the 6-month timepoint and 59.72% at the 12-month timepoint for the placebo group. In addition, for the primary outcome, pocket closure at the 12-month timepoint, a statistically significant effect was demonstrated in favor of the test group when compared with the placebo group ($P < 0.05$). Hence, the hypothesis of the study could be confirmed.

In our study, patients' level of hygiene improved markedly during the SPT. The intra-group analysis showed a statistically significant reduction in FMPS at the 12-month timepoint, compared to the baseline in all three groups (Wilcoxon test, $P < 0.05$), which in turn points to the excellent compliance of the patients.

In a previous study, the authors tested the probability of pocket closure after using locally delivered doxycycline as an adjunct to subgingival re-instrumentation [56]. The estimated probability for a site to reach the successful treatment endpoint of pocket closure (defined in the study as PPD ≤ 4 mm regardless BOP) was 45% at 3 months and 53% at 9 months for the test sites, compared to 46% and 45%, respectively, for the control sites. In the present study, the frequency of conversion of residual deep sites to sites with shallow probing depth (PPD ≤ 4 mm) attained 80.00% at 3 months and 76.25% at 9 months for test group, and 63.89% at 3 months and 63.89% at 9 months for placebo group, respectively. Previous studies [56] reported that the probability of pocket closure was not improved by the adjunctive topical doxycycline therapy. However, in our study, a statistically significant effect was demonstrated in favor of the test group, when compared to the placebo group at the 12-month timepoint. In the same study, the test group showed a mean PPD reduction of 1.1 mm after 9 months, which is consistent with our results. An additional benefit of 0.91 ± 0.42 mm was attained after 9 months in the test group and was maintained at the 12-month timepoint (0.81 ± 0.38) but was not statistically significant.

Our study revealed that repeated short USI during periodontal maintenance, with or without single adjunctive administration of antimicrobials, resulted in statistically significant improvements in mean CAL in all three groups at all timepoints, compared to baseline ($P < 0.05$). The inter-group analysis showed minor CAL improvements in favor of the Perisolv® group, when compared with both CHX and placebo groups (mean change, 0.70 ± 0.40 mm vs. 0.39 ± 0.38 mm and vs. 0.57 ± 0.50 mm at the 12-month timepoint). However, no statistically significant differences were found, except for the CAL changes in the Perisolv® group compared to the CHX group at the 6-month re-timepoint evaluation ($P = 0.0026$). These results are consistent with those reported in a previous clinical study with repeated topical administration of Perisolv® in 32 patients with at least 3 months of SPT [44]. The authors reported clinically relevant CAL gain and PD reduction of 1 mm in 1 year, without inducing further recession after 3 repeated short (1 min) USI with adjunctive administration of the antimicrobial product. Despite the measured improvements, no statistically significant difference was observed between the test and control (USI only). These results appear to suggest that a single topical administration of Perisolv® during periodontal re-instrumentation is sufficient to induce a clinically measurable effect.

It is important to consider that the participants of this study presented residual periodontal pockets, following active periodontal treatment consisting of nonsurgical or/and surgical therapy. A previous study [44] has suggested that the persistence of the pockets was caused by incomplete removal of microbial deposits during nonsurgical therapy.

Another study reporting on 202 periodontal maintenance participants (minimum of 6 months of SPT) with recurrent or persistent pockets, treated using USI (with [test] or without [control]). Participants received a slow-released doxycycline (SRD) in all residual periodontal pockets of > 4 mm [6]. Although the patients received a full cycle of periodontal therapy with periodontal surgery if indicated, a single topical administration of SRD caused a modest adjunctive benefit for 3 months only. These differences may on one hand be explained by differences in baseline PPD values (i.e., in the aforementioned study the PPD values measured ≥ 5 mm at baseline while in the present study the baseline values measured at least PPD ≥ 4 mm with BOP(+). One the other hand, the results might have also been influenced by the locally applied materials (i.e., SRD, Perisolv® and CHX, respectively).

Findings of a previous "in-vitro" study revealed that cell survival and repopulation of root surfaces is possible following either air polishing or application with Perisolv®. Moreover, it has been also shown that Perisolv® clearly reduces the vitality of the microorganisms despite failing to completely eliminate the biofilm [63]. Thus, the present study used air polishing only supragingivally to avoid influencing the outcomes of the use of Perisolv® in pockets deeper than 4 mm. At this point, it is important to mention that a statistically significant CAL gain was measured event after 6 months which in turn, points to the potential clinical relevance of using Perisolv® in residual pockets in patients enrolled in SPT.

The choice of the PCR method in the context of the currently accepted host-mediated dysbiosis of the subgingival microbiota associated with the exaggerated host response was based on the finding that recolonization by the key stone pathogen *P.g.* might play an important role in the pathogenesis of recurrent periodontitis during SPT [64] while other microbiological assessments of patients under SPT focused on the same bacteria as in our study [32, 44].The microbiological results showed no statistically significant differences among the groups at any timepoint. The intra-group comparison revealed a significant decrease in detection scores between baseline and the 12-month timepoint for *P.g.*, *P.i.*, *T.f.*, and *T.d.*. A statistically significant decrease was observed in the bacterial species, which presented relatively high counts at baseline. However, this was not the situation for *A.a.* which presented low counts at baseline with low frequency detection scores. These intra-group microbiological results compare favorably with those obtained in a similar study with repeated applications of Perisolv® [44]. The authors observed a statistically significant longitudinal reduction for only *T.f.* in the test group from baseline to day 7 and for *T.d.* from baseline to month 4. This reduction in the numbers of *T.f.* seems to correlate with the constantly improved FMPS score observed in all groups during the follow-up timepoints. Like in the above-mentioned study, no inter-group statistically significant differences were observed in our study.

Time of application and the costs of the antimicrobials are other factors that should be taken into consideration, even if not specifically addressed in our study. Since the time of

application for both products seem to be similar, an eventual cost difference between the two products could be compensated in time by the reduction of the number of residual pockets, as shown by our results in the Perisolv group. This may lead to fewer sites in need of re-instrumentation during the continuous care follow-up appointments.

Conclusion

Within their limits, the present results suggest that in patients treated for stage III–IV periodontitis and enrolled in SPT, treatment of residual pockets by means of subgingival USI and a single application of a sodium hypochlorite gel may lead to substantial clinical benefits evidenced by pocket closure.

Author contribution Conceptualization: Viorelia Radulescu, Stefan-Ioan Stratul; methodology: Darian Rusu, Giorgios Kardaras; formal analysis and investigation: Viorelia Radulescu; writing—original draft preparation: Marius Ion Boariu, Vincenzo Iorio Siciliano, Octavia Vela; writing—review and editing: Alexandra Roman, Petra Surlin, Andreea Cristiana Didilescu; supervision: Stefan-Ioan Stratul, Anton Sculean, Holger Jentsch, Luca Ramaglia.Viorelia Radulescu and Darian Rusu have equally contributed and can be both considered as first authors. All authors commented on previous versions of the manuscript, read, and approved the final manuscript.

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Declarations

Ethics approval The study was approved by the Research Ethics Committee of the Victor Babes University of Medicine and Pharmacy Timisoara (approval no.1/21.01.2018).

Informed consent All study participants provided written informed consent.

Conflict of interest The authors declare conflict of interest.

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ORIGINAL ARTICLE

Changes in clinical parameters following adjunctive local sodium hypochlorite gel in minimally invasive nonsurgical therapy (MINST) of periodontal pockets: a 6-month randomized controlled clinical trial

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Abstract

Background The mechanical disruption and removal of the subgingival biofilm represent the most important step in the treatment of periodontitis. However, in deep periodontal pockets, mechanical removal of the subgingival biofilm is difficult and frequently incomplete. Preliminary findings indicate that the use of amino acid buffered sodium hypochlorite (NaOCl) gel may chemically destroy the bacterial biofilm and facilitate its mechanical removal.

Objectives To clinically evaluate the efficacy of minimally invasive nonsurgical therapy (MINST) of periodontal pockets with or without local application of an amino acid buffered sodium hypochlorite (NaOCl) gel.

Materials and methods Forty untreated patients diagnosed with severe/advanced periodontitis (i.e. stage III/IV) with a slow/moderate rate of progression (i.e. grade A/B) were randomly allocated in two treatment groups. In the test group, the periodontal pockets were treated by means of MINST and NaOCl gel application, while in the control group, treatment consisted of MINST alone. Full-mouth plaque scores (FMPS), full-mouth bleeding scores (FMBS), probing depths (PD), clinical attachment levels (CAL) and gingival recessions (GR) were assessed at baseline and at 6 months following therapy. The primary outcome variable was PD reduction at sites with PD ≥ 5 mm at baseline.

Results At 6 months, statistically significant differences between the two groups were found ($p = 0.001$) in terms of PD and CAL change. No statistically significant differences were found in terms of GR ($p = 0.81$). The number of sites with PD ≥ 5 mm and BOP (+) decreased statistically significantly ($p = 0.001$), i.e. from 85.3 to 2.2% in the test group and from 81.6 to 7.3% in the control group, respectively. Statistically significant differences between test and control groups were recorded at 6 months ($p = 0.001$). MINST + NaOCl compared to MINST alone decreased statistically significantly ($p = 0.001$) the probability of residual PDs ≥ 5 mm with BOP− (14.5% vs 18.3%) and BOP+ (2.2% vs. 7.2%).

Conclusions Within their limits, the present results indicate that (a) the use of MINST may represent a clinically valuable approach for nonsurgical therapy and (b) the application of NaOCl gel in conjunction with MINST may additionally improve the clinical outcomes compared to the use of MINST alone.

Clinical relevance In patients with untreated periodontitis, treatment of deep pockets by means of MINST in conjunction with a NaOCl gel may represent a valuable approach to additionally improve the clinical outcomes obtained with MINST alone

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Introduction

The development and progression of periodontitis depend on the presence of pathogenic microorganisms organized in a supra/subgingival biofilm attached to the dental surface [1, 2]. The main goal of nonsurgical periodontal therapy is to eliminate the periodontal pathogenic biofilm from the tooth surfaces and from the periodontal pockets to reduce probing pocket depths and inflammation (i.e. bleeding on probing), ultimately arresting periodontal disease progression [2, 3]. Today, it is generally accepted that mechanical disruption and removal of the subgingival biofilm using hand and ultrasonic/sonic instruments represent the most important step in the treatment of periodontitis leading, in the great majority of cases, to successful clinical outcomes [2–4]. However, in certain clinical situations, such as the presence of deep periodontal pockets or deep furcation involvements, mechanical removal of the subgingival biofilm is difficult and frequently incomplete [5].

In the last years, the use of mini- and micro-instruments in combination with magnification loupes was suggested to more accurately eliminate the biofilm from deep periodontal pockets [6–8]. Clinical, microbiological and histologic findings appear to indicate that minimally invasive nonsurgical periodontal therapy may be a valuable option for the treatment of deep periodontal pockets [6–8].

Additionally, in the last decades, a number of novel strategies encompassing the use of locally delivered antiseptic and/or anti-inflammatory agents, antibiotics or photodynamic therapy, have been tested to enable a more accurate disruption and removal of the subgingival biofilm and to additionally improve the clinical outcomes and reduce the need for surgery [2, 9–11].

NaOCl has been suggested as a potential agent for the treatment of gingivitis [12] and, later, in the form of irrigation combined with mechanical debridement for the treatment of periodontitis [13].

Recently, a novel formulation consisting of NaOCl 0.95% and amino acids (glutamic acid, leucine, lysine) gel has been introduced to detoxify the root surfaces, to soften the calculus thus facilitating its removal by means of root planing [14, 15].

Findings from an "in vitro" study have shown that this novel NaOCl formulation acts have an antimicrobial effect, in particular against Gram-negative species associated with periodontitis, thus pointing to its potential use as an adjunctive topical antimicrobial in the treatment of periodontitis [14]. Subsequent findings from "in vitro" studies have shown that the application of the amino acid buffered hypochlorite solution had a positive effect on the survival, attachment and spreading of periodontal ligament cells onto root surfaces [15].

However, at present, the data on the potential clinical relevance of a local application of NaOCl used in conjunction with subgingival mechanical instrumentation is still limited [13, 16].

More recently, a novel protocol termed minimally invasive nonsurgical therapy (MINST) has been proposed for the treatment of isolated deep pockets associated with intrabony defects [17, 18]. Treatment of deep periodontal pockets by means of MINST consists of careful scaling and root planing using ultrasonic devices with delicate tips, mini-curettes and operating microscope under local anaesthesia [17, 18]. In a first study, the authors have treated intrabony periodontal defects with either MINST or minimally invasive surgical technique (MIST) [17]. The results at 3 and 6 months have failed to show any differences in terms of the clinical outcomes between the 2 procedures, thus suggesting that MINST may represent a valuable alternative to a surgical approach. An important observation was also the fact that treatment with MINST has led to an additional reduction of treatment chair time compared to MIST. A follow-up evaluation of the same patient population, together with findings made by other groups, has provided additional evidence suggesting that MINST may represent a valuable modality to successfully treat deep periodontal pockets associated with intrabony defects [18–20].

However, at present, according to the best of our knowledge, no data from randomized, controlled clinical studies are available evaluating the efficacy of MINST used with or without local application of an amino acid buffered sodium hypochlorite (NaOCl) gel in patients with untreated periodontitis.

Hence, the aim of the present randomized controlled clinical study was to evaluate the efficacy of minimally invasive nonsurgical debridement (MINST) of periodontal pockets with or without adjunct of amino acid buffered sodium hypochlorite (NaOCl) gel application over a period of 6 months.

Materials and methods

Study design

The study was designed as a double-arm, randomized controlled, superiority clinical trial. All periodontal pockets exhibiting probing depths (PD) of ≥ 5 mm were treated by means of MINST either alone (i.e. control group) or in combination with NaOCl gel application (i.e. test group). The study was conducted from May 2018 until December 2019. The study protocol was approved by the Commission on Research Ethics of the University of Messina (approval N°16/18).

Written informed consent was obtained from subjects and the study was conducted according to the Principles of the Declaration of Helsinki on experimentation involving human subjects. The research protocol was registered on Clinicaltrials.gov registry (registration number NCT04399187). The present trial was conducted according to the CONSORT statement (http://www.consort-statement.org). The null hypothesis of no statistically significant differences in terms of PD reduction between test and control procedure for the treatment of periodontal pockets was tested.

Participants

All subjects enrolled in the study were recruited from the School of Dentistry, University of Messina, Italy. Data were collected in the same research center and then the statistical analysis was performed in the Department of Periodontology, University of Naples Federico II, Italy.

Eligibility criteria for participants

Inclusion criteria:

- Untreated patients diagnosed with severe/advanced periodontitis (i.e. stage III/IV) with slow/moderate rate of progression (i.e. grade A/B) [21]
- Age ≥ 18 years old;
- Patients with at least 10 teeth per arch;
- Presence at least of two teeth with PD ≥ 5 mm per quadrant;
- Single-rooted teeth or multi-rooted teeth without furcation involvement;

Exclusion criteria:

- Patients with systemic diseases;
- Pregnant or lactating;
- Tobacco smokers (> 10 cigarettes/day);
- Previous periodontal treatment in the last 2 years;
- Prolonged antibiotic treatment or anti-inflammatory treatment within 6 months prior to periodontal therapy;
- Furcation involvement;
- Acute periodontal or endodontic abscesses;
- Third molars

Interventions

Clinical procedure

In the first session, all patients received a full-mouth supragingival scaling in order to remove the supragingival biofilm and calculus in combination with oral hygiene instructions and motivation.

After 1 week all clinical parameters were recorded (Fig. 1a) and subjects were randomly assigned to the test or control procedures. The test group was treated as follows:

1) After local anaesthesia, an amino acid–buffered sodium hypochlorite gel (Perisolv®, Regedent AG, Zurich, Switzerland) was applied for 30 s in periodontal pockets with PD ³5 mm using a sterile syringe with a plastic needle. The tip was carefully inserted into the pocket until resistance was reached and was followed by its slow ejection (Fig. 1b). No rinsing was performed after the application of the gel.
2) MINST was performed by means of careful subgingival debridement using ultrasonic scalers with specific thin tips (Instrument PS®EMS Electro Medical System S.A., Nyon, Switzerland) (Fig. 1c) and Gracey micro-curettes (Hu-Friedy®, Chicago, IL, USA) in order to minimize the trauma for the soft tissues (Fig. 1d).
3) Application of amino acid buffered sodium hypochlorite gel and MINST was performed according to the manufacturer's instructions.

In the control group, treatment consisted of MINST alone without gel application.

All treatments were performed using × 4.0 magnification loupes (Univet®, Italy). At the end of the subgingival treatment, in both groups, full-mouth supragingival cleaning by means of a rubber cup and a polishing paste was performed. Patients were instructed to rinse twice daily with 0.12% chlorhexidine digluconate (Curasept ADS® Curaden AG, Kriens, Switzerland) for the first 2 weeks. No antibiotics were prescribed. Patients were recalled on a monthly basis for professional supragingival tooth cleaning and motivation during the entire study period of 6 months when the final evaluation was made.

Outcome measures

The primary outcome variable was the probing depth (PD) reduction, defined as the distance from the gingival margin to the bottom of the pocket.

The secondary outcome variables were full-mouth plaque score (FMPS): percentage of tooth sites revealing the presence of plaque [22]; full-mouth bleeding score (FMBS): percentage of sites with bleeding on probing (BOP) [23]; clinical attachment level (CAL): distance from the cement-enamel junction (CEJ) to the bottom of the pocket and gingival recession (GR): distance from the gingival margin to the CEJ.

All clinical parameters were recorded at 6 sites per tooth by means of a manual periodontal probe (PCP-UNC 15®, Hu-Friedy, Chicago, IL, USA), applying a probing force of 0.2 N.

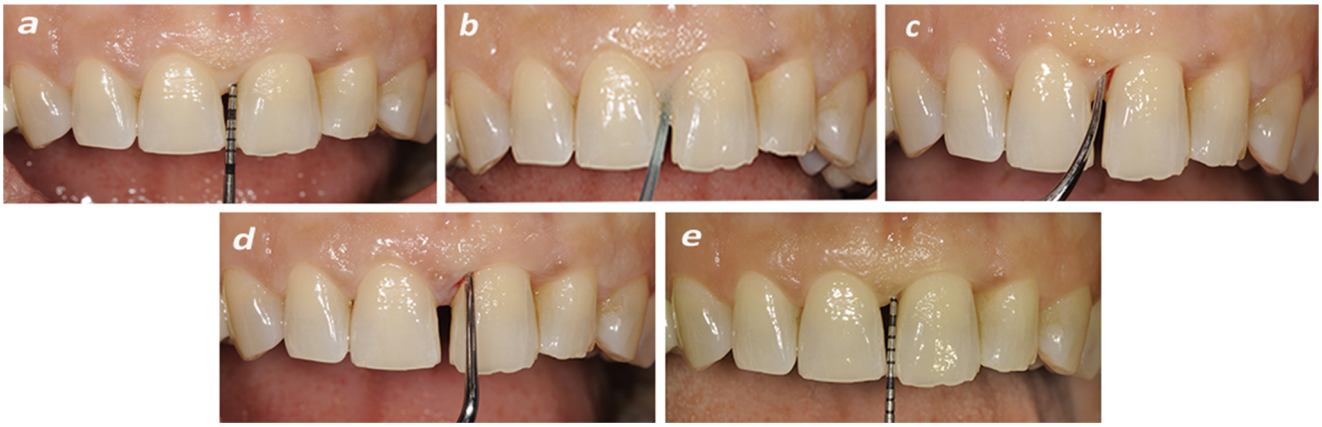

Fig. 1 **a** A probing depth (PD) of 7 mm was recorded at baseline. **b** Prior to mechanical instrumentation the NaOCl gel was applied in the periodontal pocket for 30 s. **c** Subgingival debridement was performed using an ultrasonic scaler with a thin tip. **d** A gently root planning was made by means of Gracey micro-curette. **e** A probing depth of 3 mm was recorded at 6 months post-therapy

All variables were recorded at baseline and after 6 months (Fig. 1e).

Sample size calculation

The present study was designed to test a continuous response variable (i.e. PD) from independent control and experimental subjects with 1 control per experimental subject. In a previous study, using a similar design [24], the response within each subject group was normally distributed with a standard deviation of 0.7 mm. If the true difference in the means of the experimental and control group is 0.9 mm, a sample of 22 patients (11 patients per group) is needed to reject the null hypothesis that the population means of the experimental and control groups are equal with probability (power) 0.8. The type I error probability associated with this test of this null hypothesis is 0.05. In order to compensate for patients' dropouts during the study period, a total of 40 subjects (i.e. 20 test and 20 control subjects) were enrolled in the study.

Randomization

A computerized random number generator was used in order to random assign the subjects to experimental or control procedures. A simple randomization without restrictions was done. The allocation concealment was made associating even numbers to the test procedure and odd number to the control procedure. The cards with numbers were closed in opaque envelopes and treatment allocation was performed at the time of minimally invasive nonsurgical treatment by opening the envelope containing the number.

The random allocation sequence was generated by A.B., while participants were enrolled by I.G. in the School of Dentistry, University of Messina, Italy.

Blinding and calibration

All patients enrolled in the study received periodontal therapy by the same periodontist (VIS). All parameters were recorded at baseline and after 6 months by 2 calibrated and masked examiners (I.G. and A.B.). Examiners attended a single training and calibration session on a total of 20 patients (kappa coefficient = 0.81). The calibration of all parameters was made in the same visit. The calibration meeting was performed at the School of Dentistry, University of Messina, Italy. Patients were not masked in respect to test and control procedures.

Statistical analysis

The data analysis was performed using a commercially available statistical software (NCSS-PASS, NCSS, Kaysville, UT). The patient was considered as the statistical unit; however, an additional site-based analysis was also performed. All variables were expressed in millimetres with the exception of the FMPS and FMBS, which were reported in percentage.

Descriptive statistics (e.g. mean and standard deviation) were used to present the variables (e.g. FMPS, FMBS, PD, CAL and GR). For the statistical analysis, sites with PD ≥ 5 mm at baseline were considered. An unpaired *t*-test was applied to compare the mean age of participant at baseline. A chi-square test was used to compare gender and number of smokers. In addition, also the number and percentages of sites with PD ≥ 5 with BOP positive at baseline and after the 6-month follow-up period were compared using a chi-square test.

In order to avoid pseudo-replication, an average of data proceeding from the same patient was calculated and used for statistical analysis. An intra-group comparison was made with paired *t*-test between FMPS, FMBS, PD, CAL and GR values assessed at baseline and follow-up for both procedures (i.e. MINST + NaOCl gel and MINST alone). An inter-group comparison between test and control procedures was performed with

an independent *t*-test for FMPS, FMBS, PD, CAL and GR at baseline, follow-up and for variations between baseline and follow-up values. In order to compare the frequency distribution of sites with residual PD between test and control groups, the Mantel-Haenszel χ^2 test was used. In addition, a sub-analysis for distribution of treated teeth in each group (i.e. anterior *vs* posterior and maxillary teeth *vs* mandibular teeth) was performed by means of the Mantel-Haenszel χ^2 test.

Cohen's *D* was calculated to assess the effect size in mean differences between the treatment groups for changes in PD, CAL and GR.

A p value < 0.05 was set to accept a statistically significant difference.

Results

Participants and recruitment

Figure 2 illustrates the flow chart of the study. After screening, 40 patients fulfilling the inclusion criteria were recruited. At 6 months, a total of 3 patients were lost (dropouts). Two patients were lost in the test group (subjects moved to another town). In the control group, 1 patient was lost because she was pregnant. Therefore, a total of 37 patients (18 subjects for the test group and 19 for the control group) were available for the final examination (Fig. 2). The study was conducted from May 2018 till December 2019. No complications related to any of the two procedures were recorded. Patient recruitment and treatment started in May 2018 and was completed in December 2018. The last follow-up visit was completed in June 2019. Data analysis was performed in September 2019.

Demographic characteristics

The characteristics of the patient population are presented in Table 1. Six males and 12 females (mean age 53.3 ± 9.8 years; range age 40–67 years) were included in the test group and 10 males and 9 females (48.5 ± 6.5 years; range age 36–63 years) were allocated to the control group. A total of 8 patients were smokers (< 10 cigarettes/day). No statistically significant differences ($p > 0.05$) were observed with respect to mean age, gender and smoking habits between the test and control group (Table 1).

Changes in FMPS and FMBS

Table 2 reports FMPS and FMBS at baseline and after 6-month follow-up. At baseline, FMPS was 47.1 ± 16.5% for the test group and 50.9 ± 12.4% for the control group, respectively. No statistically significant difference was found (p = 0.43) between groups. At a 6-month follow-up, a FMPS of 17.0 ± 4.8% and 17.6 ± 5.7% was recorded for the test and control group, respectively. No statistically significant differences were recorded (p = 0.72) between the test and control group. In both groups, a statistically significant change was found in terms of FMPS between baseline and 6-month follow-up (p = 0.001). At 6 months, a statistically significant improvement in mean FMBS was measured in both groups, i.e. from 39.8 ± 15.1 to 13.3 ± 6.0% in the test and from 43.8 ± 11.5 to 15.2 ± 6.0% in the control (p = 0.001) group, respectively. However, between the two groups, no statistically significant differences were found in terms of FMBS at baseline (p = 0.36) and at the 6-month follow-up (p = 0.35) (Table 2).

Probing depth changes

After 6 months, PD decreased statistically significantly (p = 0.001) from 5.96 ± 1.07 to 3.46 ± 1.08 mm in the test group and from 6.01 ± 1.60 to 4.03 ± 1.74 mm in the control group, respectively. At baseline, no statistically significant differences between the two groups (5.96 ± 1.07 mm vs. 6.01 ± 1.60 mm) were noted (p = 0.50). At 6 months, a statistically significant difference (3.46 ± 1.08 mm vs. 4.03 ± 1.74 mm) was found, favouring the test group (p = 0.001). At 6 months, the comparison between the mean changes between the test group (2.49 ± 0.76 mm) and the control group (1.98 ± 0.80 mm) was statistically significant (p = 0.001) (Table 3). The effect size (Cohen's *D*) of the PD changes from baseline to 6 months between two groups was d = 0.66 (CI 0.55–0.76).

Clinical attachment level changes

Six months after therapy, mean CAL changed from 6.24 ± 1.21 to 3.40 ± 2.16 mm in the test and from 6.41 ± 2.21 to 4.41 ± 3.02 mm in the control group, respectively. In both groups, a statistically significant difference was measured (p = 0.001). The inter-group comparison revealed a statistically not significant difference (p = 0.06) at baseline but yielded a statistically significant difference (p = 0.001) at 6 months (Table 3). The effect size (Cohen's *D*) of the CAL changes from baseline to 6 months between two groups was d = 0.42 (CI 0.32–0.52).

Gingival recession changes

The mean GR increased from 0.47±1.22 to 0.78 ± 1.72 mm in the test group and from 0.50 ± 1.33 to 0.76 ± 1.78 mm in the control group. However, the increase in GR from baseline to 6 months was not statistically significant in any of the 2 groups (p = 0.81). Furthermore, there were no statistically significant differences (p = 0.73) between the two groups at baseline and at 6 months (p = 0.81) (Table 3). The effect size (Cohen's *D*) of the GR changes from baseline to 6 months between two groups was d = 0.04 (CI − 0.06–0.13).

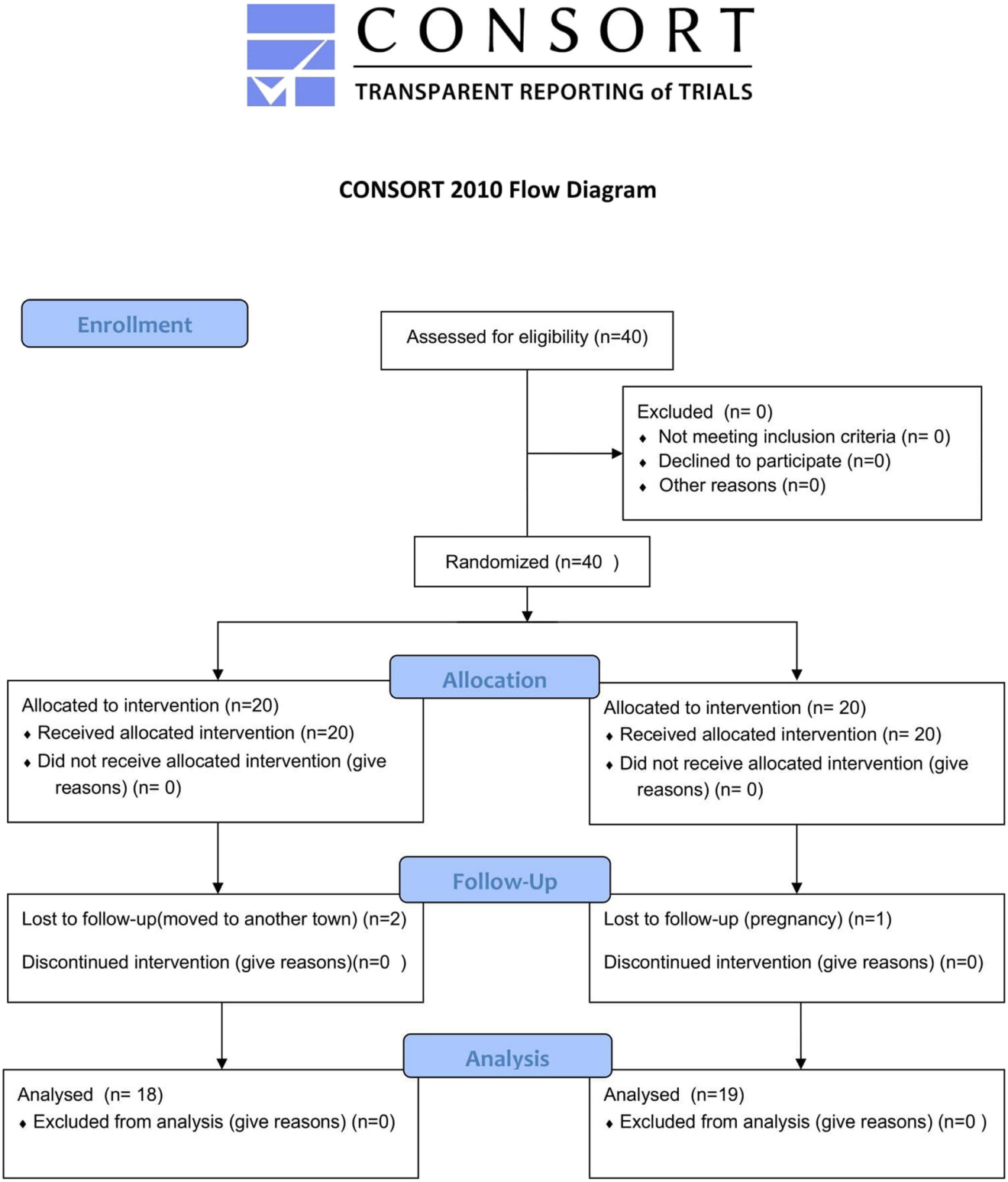

Fig. 2 CONSORT flowchart

Table 1 Patient population at baseline

	Test group ($N = 18$)	Control group ($N = 19$)	Significance (p)
Mean age (years)	53.3 ± 9.8	48.5 ± 6.5	0.43*
Range age (years)	40–67	36–63	
Gender (M/F)	6/12	10/9	0.19**
Smokers (N/%)	4; 22.2	4; 21.1	0.62**

M, male; *F*, female; *N*, number of patients

*Based on unpaired *t*-test

**Based on chi-square test

Number and percentages of sites with PD ≥ 5 mm with BOP positive

Table 4 summarized the number and percentages of sites with PD ≥ 5 mm with BOP. The number of sites with PD ≥ 5 mm and BOP decreased significantly ($p = 0.001$) from 763 (85.3%) to 20 (2.2%) for patients treated by means of MINST + NaOCl and from 594 (81.6%) to 53 (7.3%) for patients treated by means of MINST alone after 6-month follow-up. No statistically significant difference was recorded at baseline between the test and control group ($p = 0.05$). However, at 6 months, the differences between the two groups were statistically significant ($p = 0.001$) (Table 5).

Frequency distribution of residual PD

Details of the frequency distributions of residual PD changes are illustrated in Table 5. Statistically significant differences were found in terms of residual PD without BOP and for BOP-positive sites in both groups ($p = 0.001$).

In the test group, 14.5% of sites displayed PD ≥ 5 mm without BOP, while the corresponding values were 18.3% in the control group. The percentage of sites with PD ≥ 5 mm with BOP amounted to 7.2% in patients treated by means of MINST alone with the corresponding value of 2.2% sites with PD = 5 mm with BOP positive in patients treated with MINST + NaOCl. No sites with PD > 5 mm and BOP positive were found in the test group (Table 5).

Table 2 Comparison of FMPS and FMBS at baseline and after 6-month follow-up

	Baseline	6 months	Significance (p)
FMPS (%)			
Test group	47.1 ± 16.5	17.0 ± 4.8	0.001**
Control group	50.9 ± 12.4	17.6 ± 5.7	0.001**
Significance (p)	0.43*	0.72*	
FMBS (%)			
Test group	39.8 ± 15.1	13.3 ± 6.0	0.001**
Control group	43.8 ± 11.5	15.2 ± 6.0	0.001**
Significance (p)	0.36*	0.35*	

FMPS, full-mouth plaque score; *FMBS*, full-mouth bleeding score

*Based on paired *t*-test

**Based on independent *t*-test

Frequency distribution of sites with residual PD with BOP positive (*N*/%) after 6-month follow-up in respect to teeth location

A sub-analysis for the distribution of sites with residual PD with BOP positive is reported in Table 6. In anterior and posterior teeth, statistically significant differences were recorded comparing MINST + NaOCl and MINST alone ($p = 0.001$). Likewise, a statistically significant difference was found when in maxillary and mandibular sites test and control procedures were compared ($p = 0.001$) (Table 6).

Discussion

The present randomized controlled clinical trial has evaluated the outcomes obtained at 6 months by means of MINST with and without application of NaOCl in patients with untreated periodontitis exhibiting deep periodontal pockets. Both groups received exactly the same type of mechanical treatment (i.e. MINST), the only difference being the application of NaOCl in the test group prior to mechanical debridement. All pockets exhibiting probing depths (PD) of ≥ 4 mm were treated by MINST, but only pockets with PD ≥ 5 mm were considered for the statistical analysis.

At 6 months, PD decreased statistically significantly in the test group and control group, respectively. A closer analysis of the results revealed that the number of sites with PD ≥ 5 mm exhibiting BOP decreased statistically significantly in both groups, indicating excellent clinical outcomes. The obtained clinical outcomes can, on the one hand, be explained by the use of MINST consisting of careful subgingival debridement by means of ultrasonic scalers with specially designed thin tips and micro-curettes using high-magnification loupes. These findings are supported by results from previous studies,

Table 3 Comparison of probing depth (PD), clinical attachment level (CAL) and gingival recession (GR) at baseline and after the 6-month follow-up period

	Baseline	6 months	Changes	Significance (*p*)
PD (mm)				
Test group	5.96 ± 1.07	3.46 ± 1.08	2.49 ± 0.76	0.001**
Control group	6.01 ± 1.60	4.03 ± 1.74	1.98 ± 0.80	0.001**
Significance (*p*)	0.50*	0.001*	0.001*	
CAL (mm)				
Test group	6.24 ± 1.21	3.40 ± 2.16	2.84 ± 2.09	0.001**
Control group	6.41 ± 2.21	4.41 ± 3.02	2.01 ± 1.83	0.001**
Significance (*p*)	0.06*	0.001*	0.001*	
GR (mm)				
Test group	0.47 ± 1.22	0.78 ± 1.72	0.30 ± 1.16	0.81**
Control group	0.50 ± 1.33	0.76 ± 1.78	0.26 ± 0.97	0.81**
Significance (*p*)	0.73*	0.81*	0.42*	

PD, probing depth; *CAL*, clinical attachment level; *GR*, gingival recession

*Based on paired *t*-test

**Based on independent *t*-test

which have shown that MINST enables a thorough biofilm removal from the root surfaces and the periodontal pockets, reducing to a minimum the trauma of the soft tissues [17–20]. An important finding of previous studies was that at sites exhibiting intrabony defects, the use of MINST yielded similar outcomes to the surgical approach (i.e. MIST), thus pointing to the clinical relevance of this novel nonsurgical treatment modality as an alternative to the more invasive periodontal surgery [17–19].

On the other hand, it is important to be kept in mind that all the patients included in the study exhibited a high level of oral hygiene and received rigorous periodontal maintenance consisting of oral hygiene instructions and supragingival tooth cleaning performed on a monthly basis during the entire study period of 6 months.

These findings are in line with the results of a long-term study evaluating the outcomes of preventive dental treatment in a group of carefully monitored subjects who were motivated to maintain a high standard of oral hygiene and received regular supportive periodontal therapy. Today, there is ample evidence indicating that once probing depths are reduced and periodontal infection is controlled, the incidence of caries and periodontal disease as well as tooth mortality can be reduced to a minimum and kept stable over a long-time period (i.e. 30 years) [25].

An important aspect that needs to be discussed is that despite the fact that at 6 months after therapy, a dramatic reduction in the percentages of sites with PD ≥ 5 mm was measured in both groups; the magnitude of the improvement was statistically significantly higher when NaOCl gel was also applied. These clinical results appear to support the findings from "in vitro" studies which have provided evidence for the antibacterial effect of this novel NaOCl formulation and its positive effects on the survival, attachment and spreading of periodontal ligament cells [14, 15].

The present results are somewhat controversial to those very recently reported by Megally et al. [16]. In that study, a total of 365 sites in 32 patients enrolled in periodontal

Table 4 Number and percentages of sites with PD ≥ 5 with BOP positive at baseline and after the 6-month follow-up period

	Baseline	6 months	Significance (*p*)
Test groups	763/85.3	20/2.2	0.001*
Control groups	594/81.6	53/7.3	0.001*
Significance (*p*)	0.05*	0.001*	

*Based on the chi-square test

Table 5 Frequency distribution of sites with residual PD (*N*/%) with and without BOP positive after 6-month follow-up

	0–4 mm	5 mm	6 mm	7 mm	≥ 8 mm
Residual PD with BOP negative (*N*/%)					
Test group	665/74.3	86/9.6	44/4.9	0/0	0/0
Control group	496/68.1	91/12.5	28/3.8	8/1.0	8/1.0
Significance (*p*)	0.001*				
Residual PD with BOP positive (*N*/%)					
Test group	80/8.9	20/2.2	0/0	0/0	0/0
Control group	44/6.0	30/4.1	2/0.3	1/0.1	20/2.7
Significance (*p*)	0.001*				

PD, probing depth; *BOP*, bleeding on probing; *N*, number of sites

*Based on the Mantel-Haenszel χ^2 test

Table 6 Frequency distribution of sites with residual PD with BOP positive (*N*/%) after 6-month follow-up in respect to teeth location

	0–4 mm	5 mm	6 mm	7 mm	≥ 8 mm
Residual PD with BOP positive (*N*/%)					
Anterior teeth					
Test group	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Control group	8 (27.6)	5 (17.2)	1 (3.4)	0 (0)	15 (51.7)
Significance (*p*)	0.001*				
Posterior teeth					
Test group	80 (80.0)	20 (20.0)	0 (0)	0 (0)	0 (0)
Control group	36 (52.9)	25 (36.8)	1 (1.5)	1 (1.5)	5 (3.0)
Significance (*p*)	0.001*				
Maxillary teeth					
Test group	70 (77.8)	20 (22.2)	0 (0)	0 (0)	0 (0)
Control group	33 (47.1)	21 (30.0)	0 (0)	1 (1.4)	15 (21.4)
Significance (*p*)	0.001*				
Mandibular teeth					
Test group	10 (100.0)	0 (0)	0 (0)	0 (0)	0 (0)
Control group	11 (40.7)	9 (33.3)	2 (7.4)	0 (0)	5 (18.5)
Significance (*p*)	0.02*				

PD, probing depth; *BOP*, bleeding on probing; *N*, number of sites
*Based on the Mantel-Haenszel χ^2 test

maintenance and exhibiting PD ≥ 5 mm were treated by means of repeated (i.e. at months 0, 4 and 8) subgingival debridement using ultrasonic tips, alone or with a NaOCl gel. However, at 12 months, the results have failed to reveal statistically significant differences between the 2 groups, suggesting no major advantages following the use of NaOCl gel. The discrepancy between our results and those reported by Megally et al. [16] can be explained by the use of a more accurate debridement approach (i.e. MINST) in conjunction with NaOCl in patients with untreated periodontitis. It has been repeatedly demonstrated that untreated periodontal pockets react more favourable to mechanical instrumentation compared to residual pockets in patients enrolled in maintenance [26]. Furthermore, it may also be speculated that deep pockets in patients with untreated periodontitis exhibit substantially higher amounts of biofilm and calculus, compared to patients with treated periodontitis and enrolled in maintenance. Conversely, in the present patient population, the use of NaOCl formulation might have had a higher potential to exert its antimicrobial and calculus softening properties, compared to those enrolled in the aforementioned study.

A limit of the present study can be the absence of radiographic analysis of treated sites. In a previous study [27], Nibali and co-workers reported a mean of radiographic bone level change of 2.93 mm at sites associated with intrabony defects treated by means MINST. In the present study, the radiographic evaluation was not performed because most parts of the sites with PD ≥ 5 mm were associated with supra-bony defects. In these defects, no or very limited bone gain can be expected after the treatment.

Since the healing capacity and immune response of each individual can significantly vary, the comparison of periodontal tissue response among different patients to the given clinical procedures could represent a limitation of the present study. This could be avoided by assigning test and control procedures within the same dentition. However, it would have been difficult to enrol sites with the same characteristics in terms of probing depth within the same dentition (i.e. sites with PD = 5 mm on the right side and sites with PD = 5 mm on the left side). For these reasons, the investigation was based on the patient and not on site.

Within their limits, the present results indicate that (a) the use of MINST may represent a clinically valuable approach for nonsurgical therapy and (b) the application of NaOCl gel in conjunction with MINST may additionally improve the clinical outcomes compared to the use of MINST alone.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00784-021-03841-8.

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Declarations

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Commission on Research Ethics of the University of Messina (approval N°16/18) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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A nonsurgical treatment of peri-implantitis using mechanic, antiseptic and anti-inflammatory treatment: 1 year follow-up

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Abstract

Aims: The study's aim was to assess the clinical outcome 6 and 12 months after a nonsurgical treatment of peri-implantitis per se or in conjunction with a combination of local antiseptic and anti-inflammatory treatment.

Materials and methods: Included were 69 patients with periodontitis, with 106 implants, diagnosed with peri-implantitis. Peri-implantitis was defined as radiographic bone loss ≥3 mm, probing depth (PD) ≥ 6 mm, with bleeding on probing. Group M peri-implantitis was treated with ultrasonic debridement and soft tissue curettage. Group P had additional implant surface treatment with rotatory hand piece composed of chitosan bristle, soft tissue curettage combined with application of 0.95% hypochlorite and 1 mg minocycline HCl.

Results: After 6 months, both groups demonstrated significant reduction of mean plaque index, PD, and clinical attachment level (0.71 ± 0.57, 0.81 ± 0.55; 4.77 ± 0.73 mm, 4.42 ± 0.5 mm; 5.03 ± 0.86 mm, 5.13 ± 0.73 mm; respectively) and bleeding on probing. After 6 and 12 months, group P showed significantly better PD results compared to group M. The bleeding was significantly less in group P after 12 months (15.3% ± 6.2, 25.1% ± 8.2, respectively).

Conclusions: Adjunctive treatment with local antiseptic and anti-inflammatories during mechanical phase was positively associated with inflammation reduction and connective tissue reattachment.

KEYWORDS

anti-inflammatory, chitosan, minocycline, nonsurgical treatment, peri-implantitis, slow release device

1 | INTRODUCTION

Dental implants are valid choice for lost tooth replacement due to the high survival rate; however, biological complications are not rare. The main biological complication is peri-implantitis, a plaque-associated pathological condition that occurs in tissues around dental implants, which is characterized by inflammation in the peri-implant mucosa and loss of supporting bone (Berglundh et al., 2018). Extensive bone loss might require implant explanation. The prevalence of peri-implantitis is significant, as assessed in several meta-analyses: Rakic et al. (2018) reported a rate of 18.5% at patient level and 12.8% at implant level (Rakic et al., 2018); Muñoz, Duque, Giraldo, and Manrique (2018) showed similar results with 17% at patient level and 11% at implant level (Muñoz et al., 2018); while Hashim, Cionca, Combescure, and Mombelli (2018) reported a wider range with 0–62.1% at implant level and 9.1–69% at patient level (Hashim et al., 2018).

Peri-implantitis exhibits greater tissue and bone destruction compared to periodontitis (Carcuac & Berglundh, 2014; Hiyari et al., 2018), and therefore must be treated and followed more intensively. The main goals of peri-implantitis treatment are to resolve inflammation and prevent further bone loss by decontaminating the implant surface. Treatment success is determined by no suppuration or bleeding on probing (BOP), absence of erythema and swelling, no additional bone loss, and pocket depths ≤5 mm (Berglundh et al., 2018). Treatment modalities are comprised surgical and nonsurgical procedures.

Surgical procedures range between flap surgery with or without osseous resection, to regenerative approaches using xenografts, allografts, or alloplastic materials (Keeve et al., 2019; Ramanauskaite, Becker, Juodzbalys, & Schwarz, 2018). Surgical treatments are associated with risks, adverse events, and postsurgical complications. The results of surgical treatment for peri-implantitis are controversial in current literature (Chan, Lin, Suarez, MacEachern, & Wang, 2014; Keeve et al., 2019; Ramanauskaite et al., 2018).

Nonsurgical treatments include debridement using various devices (e.g., manual instruments, ultrasonic/sonic instruments, plastic or carbon tips, air powder, photodynamic therapy), with antimicrobial agents including systemic or local antimicrobial treatment (Estefanía-Fresco, García-de-la-Fuente, Egaña-Fernández-Valderrama, Bravo, & Aguirre-Zorzano, 2019; Heitz-Mayfield & Mombelli, 2014; Machtei, 2014; Suárez-López Del Amo, Yu, & Wang, 2016). Outcomes of current nonsurgical treatments show limited success and low predictability (Lang, Salvi, & Sculean, 2019).

Mechanical debridement using stainless steel instruments on implant surface causes modifications of the implant surface (Keim et al., 2019; Louropoulou, Slot, & Van der Weijden, 2012), and releases titanium (Ti) particles into the surrounding tissue (Suárez-López Del Amo, Garaicoa-Pazmiño, Fretwurst, Castilho, & Squarize, 2018), which might cause further complications (Eger, Sterer, Liron, Kohavi, & Gabet, 2017, Fretwurst, Nelson, Tarnow, Wang, & Giannobile, 2018). This requires the use of instruments to reduce implant damage while maximizing the cleaning effect (de Tapia et al., 2019; Mann, Parmar, Walmsley, & Lea, 2012; Viganò et al., 2019). In an in-vitro study, Keim et al. examined debridement with single device and found air powder abrasion was more efficient than sonic scaler, which in turn was more efficient than curette. Nevertheless, in all cases, unreached areas were visible (Keim et al., 2019). In the same study, air abrasion showed no surface damage, while sonic scaler and curette damaged the implant surface (Keim et al., 2019).

The aim of this retrospective study is to compare the clinical outcome of nonsurgical mechanical treatment of peri-implantitis, as sole treatment with a combination of mechanical, and local antiseptic and anti-inflammatory treatments, 6 and 12 months after therapy.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

This is a retrospective, single-center, clinical trial with a 12-month follow-up. The study was approved by the institutional ethical committee (0213-19-rmb) and conducted according to the principles outlined in the Declaration of Helsinki and Ethical Conduct for Research with Human Beings. Informed consents were obtained from all the subjects who participated in this study. The clinical trial is reported in accordance with Consolidated Standards of Reporting (CONSORT) guidelines.

2.2 | Study population

Subjects presented at our clinic were diagnosed with periodontitis and peri-implantitis and underwent periodontal treatment.

2.3 | Inclusion criteria

Patients with at least one titanium implant that exhibited radiographic bone loss ≥3 mm, probing depth (PD) ≥6 mm, and BOP (Berglundh et al., 2018). Periodontal treatment, including oral hygiene instruction, followed by supra and subgingival mechanical instrumentation.

2.4 | Exclusion criteria

No clinical documentation at 6 and/or 12 months post-treatment; surgery was performed on the relevant sextant.

2.5 | Treatment

Periodontal treatment consisted of supra and subgingival mechanical instrumentation of the root surface with ultrasonic instrumentation after rinsing with 0.12% CHX during 1 min, under the appropriate local anesthesia. Patients were divided according to the treatment of implants with peri-implantitis in two groups: Ultrasonic debridement with fine tips (EMS, Chemin de la Vuarpillière, 31, 1260 Nyon, Switzerland); soft tissue curettage used Teflon-coated curettes (group M), or application of 0.95% hypochlorite with amino acids (Perisolv, RLS global AB, Mölndal, Sweden) were performed. In the group P, before use, the two components were mixed together. The sodium hypochlorite and the amino acids formed short-lived chloramines (N-carboxy anhydride, NCA) in a gel consistency. The gel was syringed to the pocket and filled it until overflowed. After allowing to act for 30 s, the treatment was followed by soft tissue curettage and using rotatory hand piece composed of chitosan bristle (Labrida, Oslo, Norway). The Chitosan bristle was soaked in sterile saline for at least 2 min prior to use. This made the chitosan fibers swell, and thus became soft and flexible, leading to optimal strength. The application of the hypochlorite and the curettage were repeated three times in the session. At the end, an application of 1 mg minocycline HCl (Arestin, OraPharma, NJ) (Figure 1a–e). All patients were informed before the procedure about the two therapy modalities and they had the right to decide which treatment to choose.

All patients were seen at 3-month intervals during 1 year, as part of a routine maintenance periodontal program. Treatment outcomes were evaluated at 6 and 12 months.

2.6 | Clinical outcomes

At baseline, 6 (T1) and 12 (T2) months, the same examiner (Y.M.) recorded the following clinical variables using a manual periodontal probe (PCP-UNC 15; Hu-Friedy, Chicago, IL):

- Plaque index (PI) (Silness & Loe, 1964)
- Peri-implant (PPD), measured from the mucosal margin to the bottom of the probable pocket, and assessed at six sites per implant.
- Clinical attachment loss (CAL), measured from the implant neck to the bottom of the probable pocket, and assessed at six sites per implant.
- BOP assessed in six sites per implant.

2.7 | Radiographic examination

- Bone level (BL) was measured from the implant-abutment connection to the bottom of the bone defect by one examiner (O.G.), at

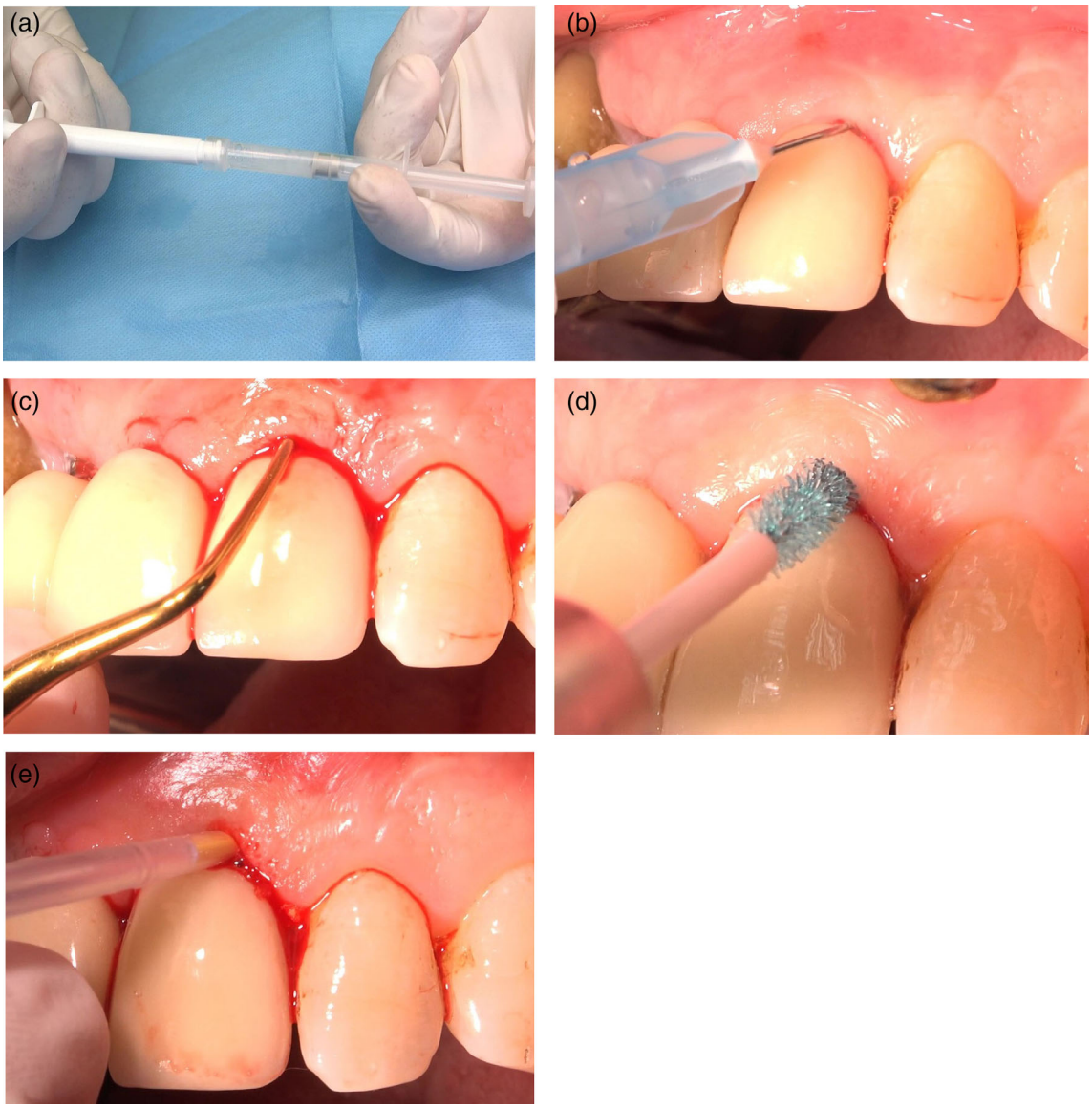

FIGURE 1 (a) Activating the solution by mixture of 0.95% sodium hypochlorite with amino acids, sodium chloride, titanium oxide, and carboxyl methylcellulose. (b) Injection of 0.95% sodium hypochlorite into the sulcus and waiting 30 s for softening the granulation tissue and prepare it for degranulation with curette. (c) Degranulation the tissue without working on the implant surface. (d) Mechanical cleaning of the implant surface with a bristle composed of a fast degrading chitosan attached to an oscillating hand piece. (e) Injection of 1 mg minocycline HCl Microspheres in to the sulcus

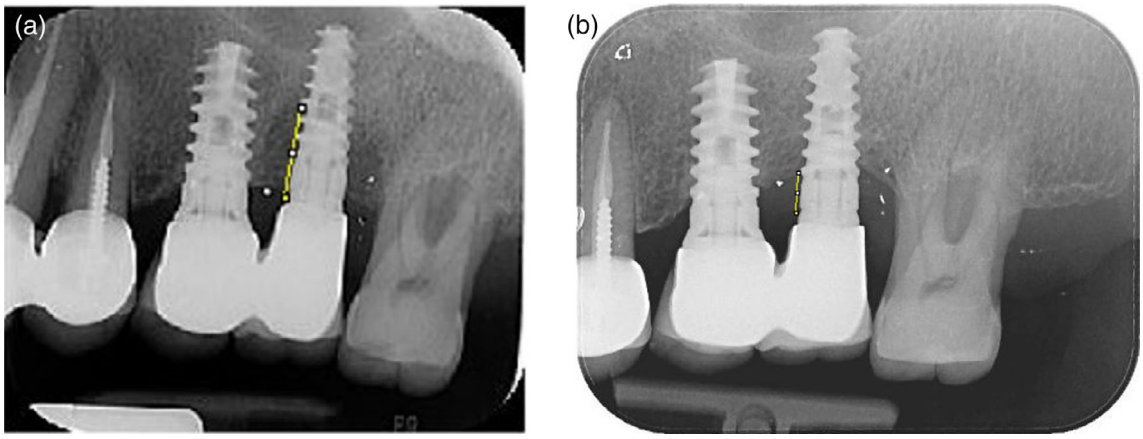

FIGURE 2 (a) Pretreatment peri-apical radiograph. (b) 12 months' post-treatment radiograph (group P)

TABLE 1 Demographic data at baseline

Characteristic	M	P
Number of patients	34	35
Number of implants	52	54
Age ± *SD*	55.3 ± 6	54.2 ± 4
Male/ female	12/22	11/24
Smoker (%)	12%	10%
Implant position		
Maxilla, (%)	46%	48%
Mandible (%)	54%	52%
Type of restoration		
Screw retained (%)	34%	39%
Cemented (%)	66%	61%

Note: Data are presented as mean (*SD*) or percentage.

baseline and T2, using image analysis software (ImageJ software, Java image processing program, National Institutes of Health [NIH], Bethesda) (Figure 2). In each radiograph, the length of the implant provided by the manufacturer was used to calibrate the "apico-coronal" measurements. The distance to the coronal bone was measured at both the mesial and distal aspects of the implant.

2.8 | Statistical analysis

Power calculation was initially performed to determine sample size. Nonsurgical therapy of peri-implantitis can reduce pocket depth 1 mm (average). Additional reduction after using antibacterial methods reach 0.7 mm, Standard values of alpha = 0.05 and power = 80% were used. Power analysis according to these parameters yielded a sample size was of at least 32 in each group.

SPSS version 19.00 software (SPSS Inc., Chicago, IL) was used for all analyses. Primary outcome was changes in PPD at the deepest site at baseline to 6 months, and baseline to 12 months. The main outcome variable (PPD changes) and secondary variables (PI and CAL) were expressed as mean ± *SD*.

Mann–Whitney *U* test was used to compare between groups among time points (time points were not normally distributed). Level of significance was set at $p = .05$.

Independent *t* tests were used to verify differences for radiographic analysis.

3 | RESULTS

Sixty nine patients treated during January 1, 2016–December 31, 2017 for periodontitis (grade 1–3, and stage A–B), who had a total of 106 implants with peri-implantitis, were included. Demographic data at baseline showed no significant differences between the two groups (Table 1).

PI, PPD, and CAL at baseline, and after 6 and 12 months, are summarized in Table 2 (mean ± *SD*). PI, PPD, and CAL decreased significantly after 6 and 12 months, compared with baseline values ($p < .001$) (Table 3). No significant differences were observed after 12 months compared to 6 months for both groups.

Comparison between the two treatments modality groups indicated a significant difference in PPD after 6 and 12 months (Table 4). With regard to PD, after 6 and 12 months group P showed significantly better results compared to group M alone (difference of 0.65 mm between baseline and 6 months and 0.64 mm between baseline and 12 months). No significant differences were found in CAL reduction between the two groups at the two time points. Bleeding was significantly reduced in the two groups after 6 and 12 months. Significantly, fewer sites with bleeding were found in group P during the entire follow-up period.

Peri-apical radiographs pretreatment and 12 months post-treatment were available for limited number of implants (12 in the group P and 15 in the group M). Radiographic analysis of bone level measurements did not yield statistically significance differences between the two treatment modalities (data not shown/data on file).

TABLE 2 Mean clinical parameters measured at baseline, 6 months, and 12 months (mean ± *SD*)

	Baseline			6 months			12 months		
	M	P	*p* value	M	P	*p* value	M	P	*p* value
PI	1.63 ± 0.65	1.51 ± 0.63	0.36	0.71 ± 0.57	0.81 ± 0.55	0.39	0.69 ± 0.5	0.78 ± 0.5	0.38
PD (mm)	6.63 ± 1.10	6.94 ± 1.32	0.19	4.77 ± 0.73	4.42 ± 0.5	**0.006**	4.90 ± 0.66	4.57 ± 0.63	**0.01**
CAL (mm)	6.87 ± 1.18	7 ± 1.38	0.59	5.03 ± 0.86	5.13 ± 0.73	0.56	5.40 ± 0.72	5.33 ± 0.67	0.60
BOP (%)	100	100	0.6	33.2 ± 12.3	21.4 ± 14.2	0.6	25.1 ± 8.2	15.3 ± 6.2	**0.05**

Note: All significant results are in bold.
Abbreviations: BOP, bleeding on probing; CAL, clinical attachment loss; PI, plaque index; PPD, probing depth.

TABLE 3 Statistical significance of periodontal parameters changes among the different time points in the same groups

	Baseline–6 months		Baseline–12 months		6–12 months	
	M	P	M	P	M	P
PI	**<0.001**	**<0.001**	**<0.001**	**<0.001**	NS	NS
PPD	**<0.001**	**<0.001**	**<0.001**	**<0.001**	NS	NS
CAL	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**
BOP	**<0.001**	**<0.001**	**<0.001**	**<0.001**	NS	NS

Note: All significant results are in bold.
Abbreviations: BOP, bleeding on probing; CAL, clinical attachment loss; PI, plaque index; PPD, probing depth.

TABLE 4 Differences between group P and M at two time points (Mann Whitney *U* test)

	Baseline–6 months	Baseline–12 months	6–12 months
PI	0.21	0.19	0.90
PPD	**0.02**	**0.019**	0.94
CAL	0.94	0.43	0.47
BOP	**0.001**	**0.001**	0.5

Note: All significant results are in bold.
Abbreviations: BOP, bleeding on probing; CAL, clinical attachment loss; PI, plaque index; PPD, probing depth.

4 | DISCUSSION

This study aimed to compare clinical findings 6 to 12 months after using two nonsurgical methods of treatment for peri-implantitis. We proposed a simple, nonsurgical treatment modality for peri-implantitis that is easily accessible and readily available for most dental practitioners. The findings revealed a positive effect of combined protocol, including nonsurgical mechanical debridement with chitosan brushes in conjunction with local delivery of minocycline microspheres and 0.95% hypochlorite buffered with amino acids; the positive effect was maintained over the 12-month follow-up period. There was a synergistic effect in combining mechanical debridement as sole treatment (improved clinical parameters) with antiseptic and anti-inflammatory treatment that further improved clinical outcome.

Biological rational of combining both materials with mechanical debridement is based on their different healing mechanisms. Hypochlorite buffered with amino acids, accompanied by mechanical debridement, disrupts the biofilm and removes granulation tissue (Roos-Jansåker, Almhöjd, & Jansson, 2017). Minocycline HCl has an antimicrobial effect, improving probing depths and bleeding scores of pathologic peri-implant tissue (Renvert, Lessem, Dahlén, Lindahl, & Svensson, 2006), and has a continuous effect, lasting for several days (Lee, Kweon, Cho, Kim, & Kim, 2018). Thus, initially removing granulation tissue and disrupting the biofilm increases efficiency of the antimicrobial agent. Furthermore, Minocycline HCl was proven to reduce collagenase activity, inhibit the activity of matrix metalloproteinases as well as osteoclast function, and thus prevent further periodontal destruction (Ingman et al., 1993; Vernillo, Ramamurthy, Golub, & Rifkin, 1994). Kivelä-Rajamäki et al. (2003) showed that the antibiotic tetracycline reduced MMP-8 (collagenase-2) in peri-implant sulcular fluid (Kivelä-Rajamäki et al., 2003).

Our results are in accordance with previous studies, although higher reduction in PD and CAL were reached when comparing group M (mechanical debridement only) (Renvert et al., 2006; Renvert, Lessem, Dahlén, Renvert, & Lindahl, 2008). This difference might be due to deeper PD and CAL at baseline examination, compared to previous studies (Renvert et al., 2006, Renvert et al., 2008). The proposed combined treatment yielded greater pocket depth reduction compared to each of the treatments (2.5 mm after 6 months, 2.37 mm after 12 months). Salvi, Persson, Heitz-Mayfield, Frei, and Lang (2007) showed improvement in PD after 6 and 12 months (1.7 and 1.7 mm, respectively) when using minocycline microspheres only (Salvi et al., 2007). Roos-Jansåker et al. (2017) showed PD reduction of 1.75 mm after 3 months, when using hypochlorite buffered with amino acids (Roos-Jansåker et al., 2017). Renvert et al. (2008) used minocycline microspheres in addition to mechanical debridement, compared to mechanical debridement only, and showed relative PD reduction of

0.6 mm after 12 months, supporting the use of minocycline. The results of our current study were similar, showing statistically significant differences in PD after 6 and 12 months (Renvert et al., 2008).

Systemic antibiotics are considered a valid approach to treat peri-implantitis, in addition to mechanical debridement (Lang et al., 2019). Mombelli and Lang (1992) showed positive clinical and microbiological results after using systemic delivery of ornidazole for 10 days, with an average PD reduction of 2.55 mm after 12 months of treatment (Mombelli & Lang, 1992). Nart et al. (2019) showed similar results using Metronidazole 500 mg every 8 hr for 7 days (Nart et al., 2019), with Liñares, Pico, Blanco, and Blanco (2019) demonstrated that adjunctive administration of systemic metronidazole has shown potential effectiveness in terms of PD and radiographic defect reduction (Liñares et al., 2019). Although it might be useful, systemic antibiotic poses some risks including: superinfection (Verdugo, 2017) and antibiotic resistance (Rams, Degener, & van Winkelhoff, 2014). Proposed protocol includes local administration of antibiotics, which reduces the risk of the above mentioned complications and achieves similar clinical results compare to administration of systemic antibiotics (average pocket depth reduction of 2.37 mm in current study).

Chitosan bristle was proved to be a safe and efficient device for debridement of dental implants (Wohlfahrt, Aass, & Koldsland, 2019; Wohlfahrt et al., 2017; Zeza, Wohlfahrt, & Pilloni, 2017). Previous studies on Chitosan bristle's added value include reduced signs of inflammation (Modified Bleeding Index [mBoP] by 1.2) and probing depth (1.15 mm) (Wohlfahrt et al., 2017, Wohlfahrt et al., 2019, Zeza et al., 2017). Chitosan is an antimicrobial that relies on numerous intrinsic and extrinsic factors, such as pH, presence or absence of metal cations, pKa, molecular weight, and degree of deacetylation (Kong, Chen, Xing, & Park, 2010). In particular, Larsen et al. (2017) found that chitosan bristle significantly reduced the amount of a periopathogenic bacteria, *Porphyromonas gingivalis* (Larsen et al., 2017). Another benefit of the chitosan bristle is its ability to reach difficult to negotiate areas, due to its flexibility and long active surface. This makes superfluous any prosthetic changes (e.g., removal of prosthetic work) as most of the prosthetic work in both groups (P, M) was cemented and not screw retained (61 and 66%, respectively).

One of the causes for peri-implantitis is residual cement, particularly in patients with history of periodontitis (Linkevicius, Puisys, Vindasiute, Linkeviciene, & Apse, 2013; Quaranta, Lim, Tang, Perrotti, & Leichter, 2017). Optional reason for the superior results of group P is cement removal, achieved in the suggested protocol in the phase of soft tissue curettage with rotatory hand piece composed of chitosan bristle. This should be further examined in future studies.

CAL did not show significant difference between the groups. This suggests that part of the improvement was due to recession of the soft tissue and part due to re-attachment of connective tissue. Extrapolating the results suggests that 1/3 of pocket reduction was due to connective tissue reattachment and 2/3 to recession formation. This improvement is in agreement with a previous study (Roos-Jansåker et al., 2017).

This study has limitations in terms of the relatively short follow-up period of 12 months; longer follow-up is required to confirm long-term results of the treatment protocol.

Another drawback is that due to the retrospective nature of this study—availability of pretreatment and 12 months' post-treatment radiographs were limited. This fact together with lack of personal stent might influence our ability to fully discover the radiographic changes following the suggested treatment modality. Therefore, future studies will include radiographic follow-up.

5 | CONCLUSIONS

Within the limitations of the present study, additional use of chitosan brush to implant surface decontamination with combined application of 0.95% hypochlorite and 1 mg minocycline HCl as part of peri-implantitis nonsurgical treatment, resulted in statistically significant clinical improvement in terms of reduction of pocket depth after 6 and 12 months.

6 | CLINICAL RELEVANCE

6.1 | Scientific rationale for study

To evaluate the clinical outcome of a nonsurgical treatment of peri-implantitis by mechanical, antiseptic, and anti-inflammatory methods; and compare it to a mechanic treatment alone.

6.2 | Principal findings

Both modalities showed improvement in clinical parameters after 6 and 12 months. Group P demonstrated greater reduction in pocket depth and bleeding.

6.3 | Practical implications

Using antiseptic and anti-inflammatory treatment during the cause related therapy at sites with peri-implantitis can be an alternative for surgery in mild to moderate cases.

CONFLICT OF INTEREST

The authors, therefore, declare no conflict of interests related to the content of this manuscript.

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Hyaluronic acid as adjunctive to non-surgical and surgical periodontal therapy: a systematic review and meta-analysis

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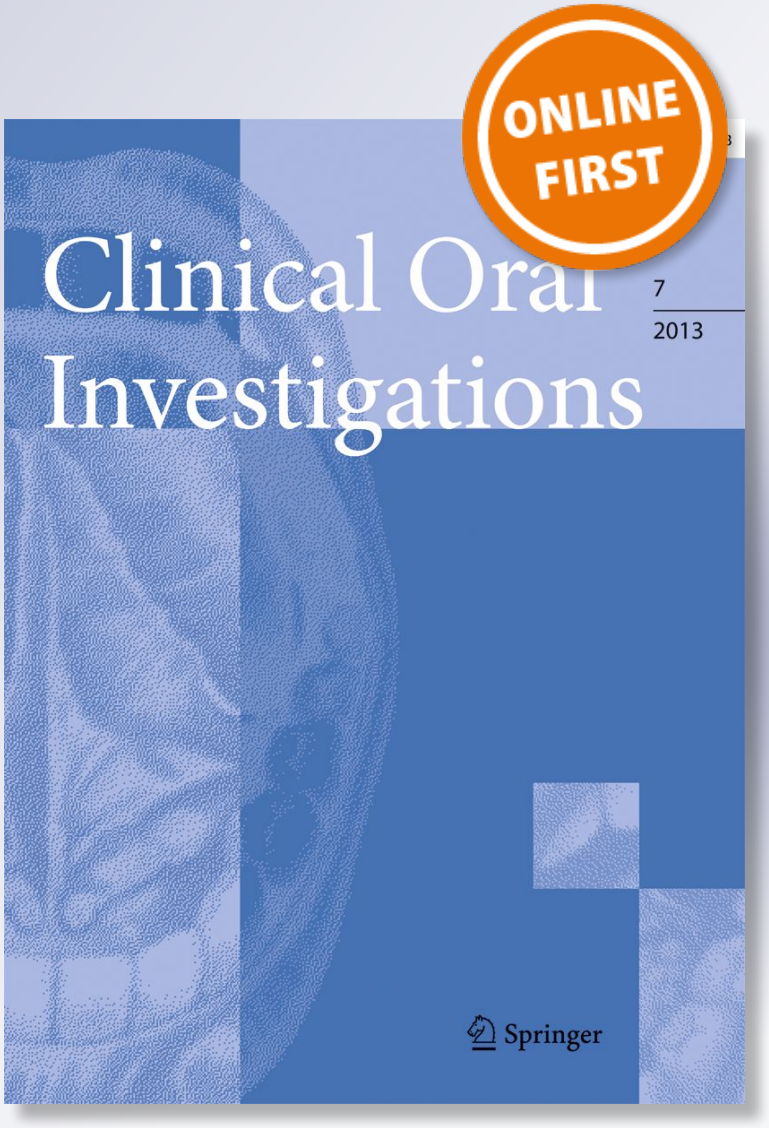

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REVIEW

Hyaluronic acid as adjunctive to non-surgical and surgical periodontal therapy: a systematic review and meta-analysis

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Abstract

Objectives To evaluate the potential added benefit of the topical application of hyaluronic acid (HA) on the clinical outcomes following non-surgical or surgical periodontal therapy.

Materials and methods A systematic search was performed in Medline, Embase, Cochrane, Web of Science, Scopus and Grey literature databases. The literature search was preformed according to PRISMA guidelines. The Cochrane risk of bias tool was used in order to assess the methodology of the included trials. Weighted mean differences (WMDs) and 95% confidence intervals (CIs) between the treatment and controls were estimated using the random-effect model for amount of bleeding on probing (BOP), probing depth (PD) reduction and clinical attachment level (CAL) gain. In order to minimize the bias and to perform meta-analysis, only randomized clinical studies (RCTs) were selected.

Results Thirteen RCTs were included: 11 on non-surgical periodontal treatment and two on surgical periodontal treatment. Overall analysis of PD reduction, CAL gain and BOP reduction in non-surgical therapy with adjunctive HA presented WMD of − 0.36 mm (95% CI − 0.54 to − 0.19 mm; $p < 0.0001$), 0.73 mm (95% CI 0.28 to 1.17 mm; $p < 0.0001$) and − 15% (95% CI − 22 to − 8%; $p < 0.001$) respectively, favouring the application of HA. The overall analysis on PD and CAL gain in surgical therapy with adjunctive HA presented WMD of − 0.89 mm (95% CI − 1.42 to − 0.36 mm; $p < 0.0001$) for PD reduction and 0.85 mm (95% CI 0.08 to 1.62 mm; $p < 0.0001$) for CAL gain after 6–24 months favouring the treatment with HA. However, comparison presented considerable heterogeneity between the non-surgical studies and a high risk of bias in general.

Conclusions Within their limits, the present data indicate that the topical application of HA may lead to additional clinical benefits when used as an adjunctive to non-surgical and surgical periodontal therapy. However, due to the high risk of bias and heterogeneity, there is a need for further well-designed RCTs to evaluate this material in various clinical scenarios.

Clinical relevance The adjunctive use of HA may improve the clinical outcomes when used in conjunction with non-surgical and surgical periodontal therapy.

Keywords Hyaluronic acid · Hyaluronan · Periodontitis · Surgical periodontal therapy · Non-surgical periodontal therapy

Introduction

Hyaluronic acid (HA) is a major natural carbohydrate component of the extracellular matrix and can be found in the skin, the joints, the eyes and most other organs and tissues including the periodontium. Furthermore, it is present in body fluids like serum, saliva and gingival crevicular fluid and as a component of the soft and hard tissues [1]. In the periodontium, HA is synthesized by HA synthase enzymes present in various cells including fibroblasts and keratinocytes in the gingival and periodontal ligament, cementoblasts and osteoblasts [2, 3].

There is evidence that HA is bacteriostatic [4, 5], fungostatic [6], anti-inflammatory [7], anti-oedematous [8], osteoinductive [7, 9–11] and pro-angiogenetic [12]. These properties suggest

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HA to be an ideal material for wound healing [13]. In animal studies, HA showed promising results for connective tissue [14, 15] and bone repair [16, 17] and it facilitated re-epithelialization, formed a good elasticity of the connective tissue and increased microvascular density when used on full thickness surgical skin wounds. The use of HA on human skin wounds and of skin ulcers resulted in faster reduction of the wound size when compared with the controls [18, 19].

Since HA is a key molecule in inflammation, granulation tissue formation, epithelium formation and tissue remodelling, it was suggested to play also an important role in periodontal wound healing [16, 20].

The above-mentioned effects (anti-inflammatory, anti-oedematous and antibacterial) have also been shown in non-surgical periodontal therapy [21]. It is anticipated that the anti-inflammatory effect is due to the exogenous HA that acts as a scavenger by draining prostaglandins, metalloproteinases and other bioactive molecule [22]. HA has shown a positive effect on the reduction of plaque and sulcus bleeding index in patients with induced gingivitis [23, 24]. In patients with chronic periodontitis, the additional application of HA to non-surgical periodontal treatment (scaling and root planing) resulted in higher clinical improvements in terms of bleeding on probing (BOP) and probing depth (PD) reduction compared with SRP alone [25]. However, other studies have failed to show statistically significant differences in terms of bacterial profile when HA was applied subgingivally as adjunctive to SRP in chronic periodontitis patients [26].

Interestingly, some clinical reports and randomized clinical trials have shown additional benefits in terms of clinical attachment level (CAL) gain and PD reduction following the adjunctive use of HA during periodontal surgery [27–29].

Most recently, one systematic review [25], however without meta-analysis, concluded that the use of HA adjunctive to SRP and to periodontal surgery yielded positive effects on the clinical outcomes (i.e. PD, CAL, BOP and bone fill). According to the best of our knowledge, at present, no meta-analysis has been published on the effects of HA when applied in the frame on non-surgical and surgical periodontal therapy. Therefore, the aim of this systematic review including meta-analysis was to evaluate the potential clinical effects of HA when used in conjunction with non-surgical and surgical periodontal therapy.

Objectives

This systematic review had the following aims:

1. To evaluate the effect of HA application on clinical parameters in conjunction with non-surgical periodontal therapy.
2. To evaluate the effect of HA application on clinical parameters as adjunctive therapy to periodontal surgery.

The PICOS (Participants, Interventions, Comparisons, Outcomes, Study Designs) research question addressing the research objectives is presented in Table 1 [30].

Materials and methods

Search method and identification of studies

Studies reporting application of HA as adjunctive to periodontal non-surgical and surgical therapies were identified by electronically searching PubMed (NCBI), Embase, Cochrane, Web of Science, Scopus and Grey literature database (www.greynet.org, https://scholar.google.ch/, www.worldcat.org) from the earliest available date through April 2016. The search strategy used was a combination of MeSH terms and/or free text words, depending on the literature database. The key words used for electronic search were "periodontics" (MeSH) OR "periodontal disease" (MeSH) OR "periodontitis" (MeSH) AND "surgical procedures, operative" (MeSH) OR "periodontal therapy" (MeSH) AND "hyaluronic acid" (MeSH) OR "hyaluronan" OR "hyaluronate" (full search strategy: Appendix 1). Hand searching of eligible article references was also performed.

Two authors (J.C.I. and M.E.) selected and evaluated independently the articles during the entire selection process, and any disagreements between authors were resolved after discussion. If information within a study should be missing, the authors would be contacted per email.

Table 1 PICOS (Participants, Interventions, Comparisons, Outcomes, Study Designs)

Participants	Chronic periodontitis patients and healthy adults
Interventions	Application of hyaluronic acid in conjunction with periodontal therapy (either surgical or non-surgical)
Comparisons	Same periodontal procedure (either surgical or non-surgical) without hyaluronic acid
Outcomes	Clinical periodontal parameters (periodontal probing depth, BOP, clinical attachment gain)
Study designs	Randomized control trials in a parallel or split-mouth design

Inclusion criteria

The study inclusion criteria were as follows:

1. Study design—randomized controlled trials (parallel- or split-mouth design).
2. English language.
3. No year restriction.
4. Studies reporting application of HA as adjunctive to non-surgical and surgical periodontal therapy.
5. No combinations with biomaterials (e.g. bone substitute, membranes).
6. Minimum 3-month follow-up period for non-surgical treatment and minimum 6-month follow-up period for surgical treatment.
7. Studies reporting either on PD, CAL or BOP as outcomes.

Type of outcome measurements

The primary outcomes were changes in PD, CAL and BOP reported at different time points.

Data collection

The following data from each study were extracted and entered into an electronic spreadsheet:

Name of the authors, year of publication, total number of participants, total amount of treated sites, months of follow-up, BOP, PD, CAL and study design.

Risk of bias (quality) assessment

The Cochrane risk of bias tool [31] was used in order to assess the methodology of the included trials. Two authors (M.E., J.C.I.) independently assessed risk of bias on the following criteria:

1. Random sequence generation.
2. Allocation concealment.
3. Blinding of participants and the investigator.
4. Blinding of outcome assessments.
5. Incomplete outcome data.
6. Selective outcome reporting.
7. Other bias.

Each relevant domain per trail was judged either as low risk (if all criteria were met), unclear risk (only one criterion was missing) or high risk (two or more criteria were missing). As a proxy to publication bias, a funnel plot and the Egger tests were applied only for non-surgical studies outcome on PD, which was considered in ten trials. For the other outcomes and the surgical studies, publication bias could not be assessed because there were fewer than ten included studies.

Data analysis

The treatment outcomes used in the meta-analysis were changes in PD, CAL and BOP from baseline and at 3 months after periodontal pockets were treated by non-surgical therapy. For surgical therapy, only PD and CAL changes were assessed after a follow-up period of at least 6 months.

A correlation coefficient of 0.5 was used in order to calculate the standard deviations (SDs) of the mean difference of the before and after outcome changes. Weighted mean differences (WMDs) and 95% confidence intervals (CIs) between the treatment and controls were estimated using the random-effect model for the continuous outcome amount of BOP, PD reduction and CAL gain. For studies providing only the interquartile ranges (ICRs), the SD was estimated by dividing the ICR by 1.35 [31].

Results were presented as forest plots with weighted means and 95% CIs. Heterogeneity across studies was evaluated using I^2 statistic ($I^2 \geq 50\%$ denoting substantial heterogeneity). All statistical analyses were conducted using the "metan" family of commands in Stata 14.2 (Stata Corp, College Station, TX, USA). Statistical significance was set at $p < 0.05$.

The alternative research hypothesis of this study was that there are differences in the treatment outcomes between the intervention group (with HA) and the control group (without HA).

Results

Search results

A total of 438 studies were identified in six databases. After elimination of duplicates, 261 studies could be assessed. Two hundred forty-three studies had to be excluded in the process of title and abstract reading. Those studies were case series, written in a language other than English, had not an appropriate follow-up or used a combination with other biomaterials (e.g. membranes, bone substitutes). Eighteen full-text publications were further assessed for eligibility. After full eligibility assessment, five studies and the surgical part of one study were excluded (Table 2) and 13 studies were included in this review (search flow diagram: Fig. 1). Among the included studies, 11 clinical trials reported on the effect of HA in non-surgical therapy (scaling and root planing) in patients with chronic periodontitis [26, 32–41] (Table 3), and two studies reported on the effect of HA as adjunct to surgical periodontal therapy [27, 28] (Table 4). The surgical studies compared either flap surgery alone or flap surgery with HA delivery into intrabony defects.

Table 2 Excluded studies and reason for exclusion

Author	Reason for exclusion
Engström et al. (2001)	Only the surgical part was excluded—using a combination of a membrane and HA
Kaira et al. (2015)	Case report + combination of HA with amnion membrane
Mesa et al. (2002)	Study focused on effect of an HA gel on cell proliferation and inflammation
Pilloni et al. (2011)	Not patients with chronic periodontitis
Sandhu et al. (2015)	Case report + combination of HA with platelet-rich fibrin
Xi et al. (2014)	Language (Chinese)

Results of meta-analyses for non-surgical therapy

CAL gain

Nine studies [26, 32–34, 36, 37, 40, 41] reported data on CAL gain for sites treated with scaling and root planing either with or without the adjuvant use of HA after 3 months. Overall, the WMD was 0.73 mm (95% CI 0.28 to 1.17 mm; $p < 0.0001$), favouring the addition of HA. However, considerable heterogeneity was identified among studies (chi-squared test $p < 0.0001$) (Fig. 2).

PD reduction

Eight studies [26, 32–34, 36–38, 41] reported data on PD reduction for sites treated with the use of HA versus a control group without. The WMD of the eight studies was −0.36 mm (95% CI −0.54 to −0.19 mm; $p < 0.0001$), favouring the treatment with HA. Considerable heterogeneity was identified among studies (chi-squared test $p < 0.0001$) (Fig. 3).

BOP reduction

Five studies [26, 32, 34, 37, 41] reported data on BOP reduction in percentage of sites treated with HA versus a control group. Overall, the WMD was −15% (95% CI −22 to −8%; $p < 0.001$), favouring the treatment. Despite, considerable heterogeneity was identified among studies (chi-squared test $p < 0.0001$) (Fig. 4).

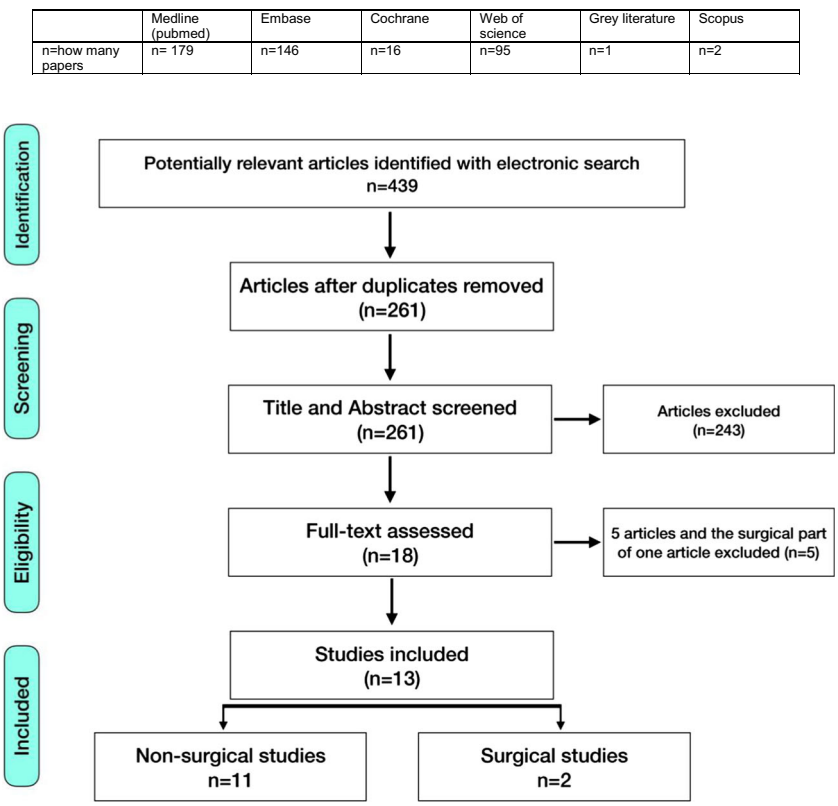

Fig. 1 Flow diagram describing the search and study inclusion process

Table 3 Characteristics of included studies—non-surgical therapy

Author (year)	Study design	Participants Control/test	Clinical parameters Control/test	Intervention	Follow-up	Outcomes	Site and funding
Bevilacqua (2012)	Split mouth	11 individuals	Average BOP (%) 72.7 72.7 Average PD (mm) 6.36 (5.86–6.87) 6.14 (5.7–6.58) Average CAL (mm) 5.91 (5–6.83) 5.91 (5–6.84)	1. SRP + placebo 2. SRP + HA	45 days 90 days	PD, CAL, BOP, PI	University, industry
Chauhan (2013)	Parallel groups	40 individuals 20/20	Average CAL (mm) 6.10 ± 0.38 6.13 ± 0.54 Average PD (mm) 5.93 ± 0.6 5.90 ± 0.27	1. SRP 2. SRP + HA	3 months	PD, CAL, GI, PI	University, industry
Eick (2013)	Parallel groups	34 individuals 17/17	Average PD (mm) 4.1 ± 0.4 mm 4.2 ± 0.4 mm Average BOP (%) 18.8 ± 11.1 mm 16.3 ± 8.7 mm Average CAL (mm) 5.7 ± 0.6 mm 5.5 ± 0.9 mm	1. SRP 2. SRP + HA + 2*d HA rinsing for 2 weeks	3 + 6 months	BOP, PI, CAL, PD	University, industry
Engström (2001)	Split mouth	9 individuals	Average PD (mm) 6.8 ± 1.5 6.4 ± 1.3	1. SRP 2. SRP + HA (3×)	2 weeks 1 month 3 months 6 months	BOP, PI, PD	University, industry
Gontiya and Galgali (2012)	Parallel groups	26 individuals 13/13	Average PD (mm) 6.42 ± 0.44 6.57 ± 0.45 Average CAL (mm) 8.56 ± 0.41 8.91 ± 0.41	1. SRP 2. SRP + HA (4×)	12 weeks	PD, CAL (RAL), PD	University
Johannsen (2009)	Split mouth	12 individuals	Average BOP (%) 58 (26) 74.5 (45.7) (IQR) Average CAL (mm) 4.5 (4.2–4.7) 4.4 (4.1–4.8) Average PD (mm) 4.2 (3.6–4.7) 4.2 (3.7–4.7)	1. SRP 2. SRP + HA (2×)	12 weeks	BOP, PI, CAL, PD	University, industry

Table 3 (continued)

Author (year)	Study design	Participants Control/test	Clinical parameters Control/test	Intervention	Follow-up	Outcomes	Site and funding
Koshal (2012)	Split mouth	52 individuals	Average PD (mm) 3.90 ± 0.93 3.82 ± 0.78	1. SRP + placebo 2. SRP + HA	3 months	GI, PD	University, industry
Polepalle (2015)	Split mouth	18 Individuals	Average PD (mm) 5.21 ± 0.54 4.99 ± 0.34 Average CAL (mm) 5.41 ± 0.65 5.40 ± 0.71	1. SRP 2. SRP + HA (2×)	12 weeks	GI, PI, PD, CAL	University
Rajan (2014)	Split mouth	33 individuals	Average PD (mm) 6.09 ± 1.26 6.33 ± 0.99 Average CAL (mm) 9.12 ± 1.67 10.18 ± 2.08	1. SRP 2. SRP + HA (2×)	4 weeks 12 weeks	GI, PI, PD, CAL	University
Wan (2004)	Parallel groups	56 individuals 28/28	Average BOP (%) 67.4% ± 21.2 71.3% ± 16.8 Average PD (mm) 2.5 ± 0.7 2.4 ± 0.5 Average PAL (mm) 12.7 ± 2.6 12.3 ± 2.2	1. SRP + placebo 2. SRP + HA (2×)	1 month 3 months	BOP, PI, PD, CAL (PAL)	University, industry
Xu (2004)	Split mouth	20 individuals	Average PD (mm) 5.2 ± 1.62 5.3 ± 1.61 Average CAL (mm) 5.4 ± 1.97 5.5 ± 1.79 Average BOP (mm) 72% 78%	1. SRP 2. SRP + HA (6×)	6 weeks 12 weeks	BOP, CAL, PD	Grant (German Academic Exchange Service), university

BOP bleeding on probing, *PD* probing depth, *CAL* clinical attachment level, *PI* plaque index, *HA* hyaluronic acid, *SRP* scaling and root planing, *GI* gingival index, *RAL* relative attachment level, *PAL* probing attachment level, *IQR* interquartile range

Table 4 Characteristics of included studies—surgical therapy

Author (year)	Study design	Participants Control/test	Clinical parameters Control/test	Intervention	Follow-up	Outcomes	Site and funding
Briguglio (2013)	Parallel groups	40 individuals 20/20	Average PD 8.0 ± 0.7 8.6 ± 1.5 Average CAL 8.3 ± 1.2 7.2 ± 1.5	1. IBD + EDTA 2. IBD + EDTA + HA	12 months 24 months	PD, CAL, BOP, PI	University
Fawzy El-Sayed (2012)	Split mouth	14 individuals 2 teeth per site	Average CAL 5.50 (5.00/8.00) 5.50 (2.00/7.00) (IQR) Average PD 5.00 (5.00/6.00) 5.00 (5.00/6.00) (IQR)	1. IBD 2. IBD + HA	3 months 6 months	CAL, GR, PD, GI, PI	Funded by the first author

BOP bleeding on probing, *PD* probing depth, *CAL* clinical attachment level, *PI* plaque index, *GI* gingival index, *HA* hyaluronic acid, *GR* gingival recessions, *IBD* intrabony defect, *EDTA* ethylenediaminetetraacetic acid, *IQR* interquartile range

Results of meta-analyses for surgical therapy

CAL gain

Two studies reported data on CAL gain for sites treated with HA versus a control group at 6 months and 24 months [27, 28]. The WMD was 0.85 mm (95% CI 0.08 to 1.62 mm; $p < 0.0001$), favouring the treatment. A low heterogeneity among studies was observed (chi-squared test $p = 0.822$) (Fig. 5).

PD reduction

Two studies [27, 28] reported data on PD reduction for sites treated with HA versus a control group at 6 months and 24 months. Overall, the WMD was − 0.89 mm (95% CI − 1.42 to − 0.36 mm; $p < 0.0001$), favouring the adjunctive use of HA. Furthermore, the comparison presented low heterogeneity among the two studies (chi-squared test $p = 0.714$) (Fig. 6).

BOP reduction

BOP was not measured in the included studies. Therefore, there are no results for BOP reduction in surgical therapy.

Results of risk of bias assessment

Results of the risk of bias assessment for the included RCTs are summarized in Table 5. Only three studies were

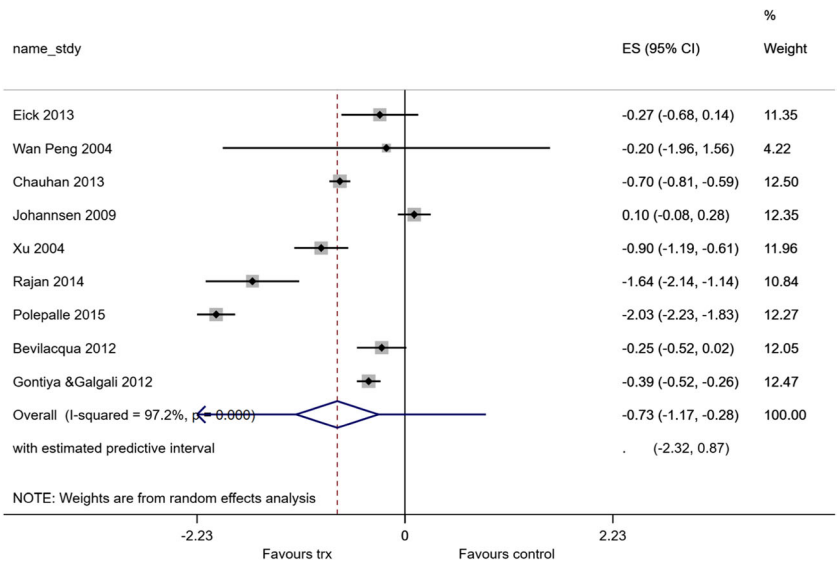

Fig. 2 Forest plots for CAL gain following non-surgical therapy after 3 months

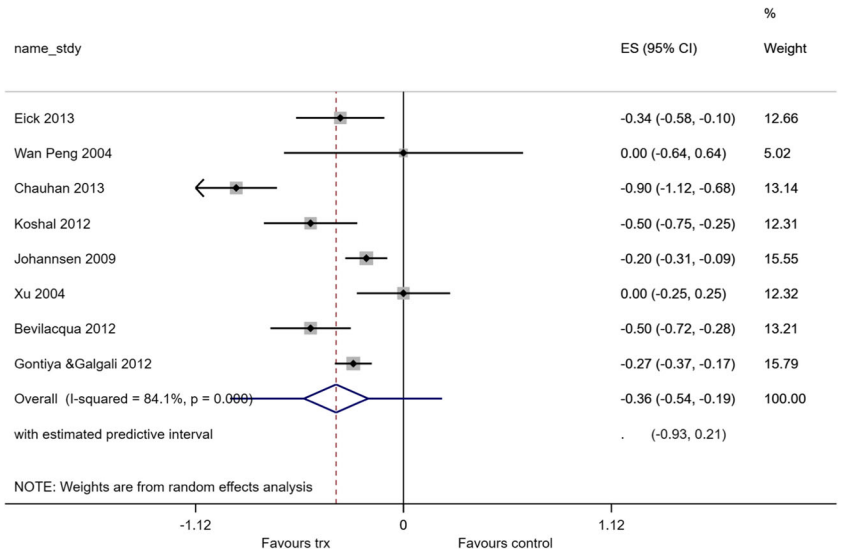

Fig. 3 Forest plot for PD reduction following non-surgical therapy after 3 months

assessed at low risk of bias while 11 studies were determined to be at high risk.

Publication bias

The Egger test was not significant suggesting that there was no evidence for small study effects. The funnel plot is asymmetric; however, it is difficult to assess whether this is due to publication bias as a number of reasons could be the reason for this asymmetry [42] (Fig. 1).

Discussion

The present systematic review including meta-analysis has evaluated the potential additional effects of local application of HA on the clinical outcomes of non-surgical and surgical periodontal therapy.

Thirteen RCTs fulfilled the inclusion criteria with adequate follow-up (3 months for non-surgical treatment and more than 6 months for surgical treatment).

Eleven RCTs have evaluated the effectiveness of HA adjunctively to non-surgical treatment on chronic periodontitis patients. Six out of the 11 studies were performed in a split-mouth design and five in a parallel group design. The application frequency of the different HA-containing products differed between the studies from one application during scaling and root planing to a repeated application during scaling and root planing and additional weekly applications up to 6 weeks.

The meta-analysis revealed that non-surgical treatment with adjunctive HA resulted in additional PD reduction (mean − 0.36 mm), CAL gain (mean 0.73 mm) and BOP reduction (mean − 15%) compared with conventional scaling and root

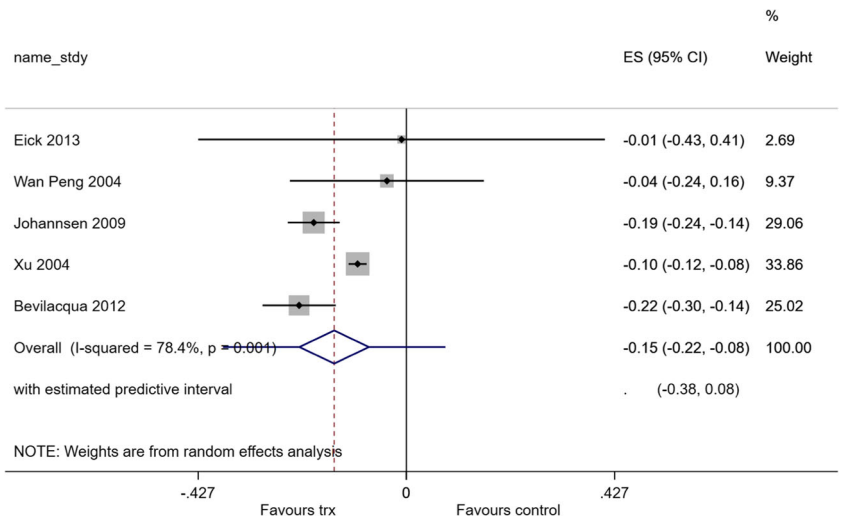

Fig. 4 Forest plot for BOP reduction following non-surgical therapy after 3 months

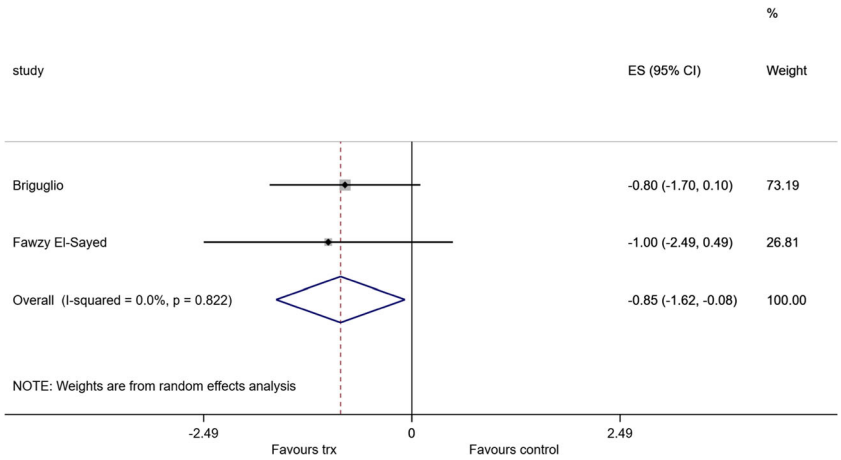

Fig. 5 Forest plot for CAL gain following surgical therapy after 6–24 months

planing after 3 months. If we are looking at results of a recently published systematic review [43] about additional CAL gain with different adjuncts compared with scaling and root planing alone (0.35 mm with systemic antimicrobials, PDT diode laser 0.53 mm, chlorhexidine chips 0.40 mm), HA could represent a suitable alternative to the most frequently used adjuvants. Nevertheless, there was an overall high risk of bias and a high heterogeneity among the studies.

The heterogeneity among the studies may be attributed to differences in the treatment protocol and the different types of products used. All the products contained high molecular weight HA with a concentration from 0.2 to 0.8%. It needs to be kept in mind that the most appropriate protocol, product and concentration for the clinical application of HA are still unknown. Moreover, in the included studies, there are different time points and different number of applications. Additionally, it is still unknown which formulation of HA (i.e. cross-linked or non-cross-linked) will give the best clinical result [44].

Two RCTs have evaluated the effectiveness of HA as an adjunctive to surgical treatment (open flap debridement (OFD)) in chronic periodontitis patients. One study was conducted as a split-mouth study and one with a parallel group design. In both studies, intrabony defects were treated with either OFD + HA (test) or OFD (control). The results have shown that after 6–24 months, the adjunctive application of HA yielded statistically significantly higher clinical improvements evidenced by PD reduction and CAL gain compared with OFD alone thus suggesting that HA has an added beneficial effect when used as an adjunct to periodontal surgery [27, 28]. It is generally accepted that angular bony defects, when left untreated, will worsen/progress over time, eventually leading to tooth loss [45]. The results of the present meta-analysis indicate that the use of HA in conjunction with OFD may provide an added clinical benefit evidenced by a further reduction in PD and gain of CAL gain in intrabony defects compared with OFD alone.

The added clinical improvements shown in the present meta-analysis are in line with the results from several preclinical and clinical studies. A case series of surgical periodontal therapy in conjunction with HA and autologous bone revealed good clinical outcome without the use of a membrane [46]. Furthermore, another case series showing promising results in intrabony defect treated with HA in conjunction with OFD

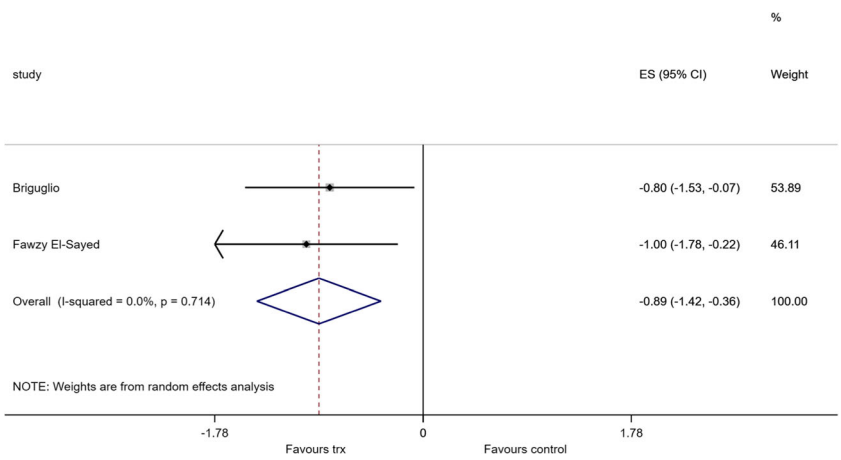

Fig. 6 Forest plot for PD reduction following surgical therapy after 6–24 months

Table 5 Results of quality assessment

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessments (detection bias)	Incomplete outcome data (attrition bias)	Selective outcome reporting (reporting bias)	Other bias	General risk assessment
Bevilacqua (2012)	+	+	+	+	+	+	+	Low
Chauhan (2013)	+	+	−	?	+	+	−	High
Eick (2013)	+	+	−	+	−	+	?	High
Engström (2012)	+	+	−	+	−	+	?	High
Gontiya and Galgali (2012)	+	+	?	?	+	+	?	High
Johannsen (2009)	+	+	−	?	+	+	?	High
Polepalle (2015)	+	+	−	?	+	+	?	High
Rajan (2014)	+	?	+	+	?	?	?	High
Wan (2004)	+	+	+	+	+	+	+	Low
Xu (2004)	+	+	−	−	+	+	?	High
Kohal (2012)	+	−	+	−	+	+	?	High
Briguglio (2013)	+	+	+	+	+	+	+	Low
Fawzy El-Sayed (2012)	+	+	−	?	+	+	?	High

'+' = low risk; '?' = unclear risk; '-' = high risk

[29]. HA has shown to increase osteoblast activity by stimulating differentiation and migration of mesenchymal cells [6] and accelerate bone formation in a rabbit model [47]. Kim et al. reported that HA improved wound healing and bone formation in hemisectioned-performed extraction sockets with communication to periodontal lesions in a canine model [48].

Taken together, the positive outcomes reported in preclinical and clinical studies corroborate the results of the present meta-analysis and lend additional support to the capacity of HA to improve wound healing. Findings from medical field have shown that HA possesses a number of properties that are relevant in wound healing such as stabilizing the blot clot, lowering the inflammatory response, helping in neovascularization and angiogenesis and accelerating fibroblast migration and wound closure [49, 50].

The above-mentioned positive biologic effects of HA are also supported by the results of a recently published preclinical (i.e. in vitro) study which have demonstrated that HA enhanced expression of genes encoding type III collagen and transforming growth factor-β3, characteristic of scarless wound healing [44]. The application of HAs up-regulated the expression of genes encoding pro-proliferative, pro-migratory and pro-inflammatory factors in palatal and gingival fibroblasts while in palatal but not gingival fibroblasts, an indirect effect of HA on the expression of matrix metalloproteinases 2 and 3 was detected. Taken together, these preclinical data provide further support on the effects of HA to enhance the proliferative, migratory and wound-healing properties of cell types involved in periodontal wound healing/regeneration.

When discussing the role of HA on wound healing, it needs to be also pointed to the findings of a preclinical study in dogs, which have failed to show an advantage of using HA in periodontal surgery [51]. Following the application of HA in surgically created class III furcation defects, the histological analysis did not reveal any substantial formation of root cementum, periodontal ligament and bone. However, these negative findings are most likely due to the low regenerative potential of class III furcation defects [52].

It has also to be realized that the present systematic review and meta-analysis has a number of limitations, and therefore, the results need to be interpreted with caution. First of all, there is a significant heterogeneity

between the studies evaluating HA in non-surgical periodontal therapy due to study design, treatment time points, products and outcome assessments. Second, out of 13 RCTs evaluating the effects of HA in conjunction with surgical periodontal therapy, only two studies fulfilled the inclusion criteria (i.e. 11 had a high risk or unclear risk of bias), and thus, there is a need for well-designed, controlled clinical studies evaluating this material in conjunction with periodontal surgery.

Obviously, due to an overall high risk of bias and heterogeneity among the studies, there is a need for future well-designed RCTs to justify the benefits of using HA for non-surgical periodontal treatment. Last but not least, an appropriate protocol and the most adequate formulation of HA for clinical applications need to be tested and further evaluated.

Conclusion

Within their limits, the present data indicate that the topical application of HA may lead to additional clinical benefits when used as an adjunctive to non-surgical and surgical periodontal therapy. However, due to the high risk of bias and heterogeneity, there is a need for further well-designed RCTs to evaluate this material in various clinical scenarios.

Funding The work was supported by the authors' own institutions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study (e.g. systematic review and meta-analysis), formal consent is not required.

Appendix 1 Full searching strategy

((“periodontics” [MeSH Terms] OR “periodontics” [All Fields] OR “periodontology” [All Fields]) OR (“periodontitis” [MeSH Terms] OR “periodontitis” [All Fields]) OR (“periodontal diseases” [MeSH Terms] OR “periodontal” [All Fields] AND “diseases” [All Fields]) OR “periodontal diseases” [All Fields] OR (“periodontal” [All Fields] AND “disease” [All Fields]) OR (“periodontal disease” [All Fields] OR “periodontal pocket” [MeSH Terms]) OR (“periodontal” [All Fields] AND “pocket” [All Fields]) OR (“periodontal pocket” [All Fields]) OR (furcation [All Fields] AND (“therapy” [Subheading] OR “therapy” [All Fields] OR “therapeutics” [MeSH Terms] OR “therapeutics” [All Fields])) OR (intrabony [All Fields] AND defect [All Fields]) OR (infrabony [All Fields] AND defect [All Fields]) OR (intraosseous [All Fields] AND defect [All Fields]) OR (periodontal [All Fields] AND (“surgery” [Subheading] OR “surgery” [All Fields] OR “surgical procedures, operative” [MeSH Terms] OR (“surgical” [All Fields] AND “procedures” [All Fields] AND “operative” [All Fields]) OR “operative surgical procedures” [All Fields] OR “surgery” [All Fields] OR “general surgery” [MeSH Terms] OR (“general” [All Fields] AND “surgery” [All Fields]) OR “general surgery” [All Fields])) OR (periodontal [All Fields] AND (“therapy” [Subheading] OR “therapy” [All Fields] OR “therapeutics” [MeSH Terms] OR “therapeutics” [All Fields])) OR (periodontal [All Fields] AND (“regeneration” [MeSH Terms] OR “regeneration” [All Fields]))) AND (hyaluron [All Fields] OR (“hyaluronic acid” [MeSH Terms] OR (“hyaluronic” [All Fields] AND “acid” [All Fields]) OR “hyaluronic acid” [All Fields]) OR (“hyaluronic acid” [MeSH Terms] OR (“hyaluronic” [All Fields] AND “acid” [All Fields]) OR “hyaluronic acid” [All Fields] OR “hyaluronan” [All Fields]) OR (“hyaluronic acid” [MeSH Terms] OR (“hyaluronic” [All Fields] AND “acid” [All Fields]) OR “hyaluronic acid” [All Fields] OR “hyaluronate” [All Fields]))

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ORIGINAL ARTICLE

Effects of air polishing and an amino acid buffered hypochlorite solution to dentin surfaces and periodontal ligament cell survival, attachment, and spreading

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Abstract
Objectives The aim of this study is to examine morphological changes of dentin surfaces following air polishing or amino acid buffered hypochlorite solution application and to assess their influence on periodontal ligament (PDL) cell survival, attachment, and spreading to dentin discs in vitro.
Materials and methods Bovine dentin discs were treated with either (i) Classic, (ii) Plus, or (iii) Perio powder (EMS). Furthermore, Perisolv® a hypochlorite solution buffered with various amino acids was investigated. Untreated dentin discs served as controls. Morphological changes to dentin discs were assessed using scanning electron microscopy (SEM). Human PDL cells were seeded onto the respectively treated discs, and samples were then investigated for PDL cell survival, attachment, and spreading using a live/dead assay, adhesion assay, and SEM imaging, respectively.
Results Both control and Perisolv®-rinsed dentin discs demonstrated smooth surfaces at low and high magnifications. The Classic powders demonstrated the thickest coating followed by the Powder Plus. The Perio powder demonstrated marked alterations of dentin discs by revealing the potential to open dentinal tubules even before rinsing. Seeding of PDL cells demonstrated an almost 100 % survival rate on all samples demonstrating very high biocompatibility for all materials. Significantly higher PDL cell numbers were observed on samples treated with the Perio powder and the Perisolv® solution (approximately 40 % more cells; $p < 0.05$). SEM imaging revealed the potential for PDL cells to attach and spread on all surfaces.
Conclusion The results from the present study demonstrate that cell survival and spreading of PDL cells on root surfaces is possible following either air polishing or application with Perisolv®. Future in vitro and animal testing is necessary to further characterize the beneficial effects of either system in a clinical setting.
Clinical relevance The use of air polishing or application with Perisolv amino acid buffered hypochlorite solution was effective in treating root surfaces and allowed for near 100 % PDL cell survival, attachment, and spreading onto all root surfaces.

Keywords Periodontal regeneration · Powder spraying · Air-Flow · Dentin discs · Dentinal tubules

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Introduction

Periodontitis is a widespread inflammatory disease of the tooth-supporting soft and hard tissues, which is modulated by the host [1]. Biofilms are regarded as the primary etiologic factor for both disease initiation and progression [2]. Therefore, any cause-related periodontal therapy is based on the strict removal of the pathogenic microbial challenge and the successful prevention of their re-establishment [3].

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Clinically, this is achieved traditionally by mechanical debridement using scalers, curettes, and/or ultrasonic instruments along with proper oral hygiene instructions [4, 5].

The preservation and creation of a biocompatible tooth surface during this periodontal therapeutic approach is crucial for successful tissue integration [6]. This is, however, particularly difficult when the surfaces display distinct morphological features, which are difficult to reach and to clean [7]. As a consequence, the overall aimed therapeutic goals are difficult to achieve and it is well documented that the deeper the initial periodontal lesions are, the less effective mechanical debridement may be [8–10].

A number of instruments have been developed and recommended over the years to assist clinicians in removing bacteria and their deposits in severely affected sites. Most of the classical mechanical instruments including curettes and ultrasonic instruments—despite being effective in hard deposit removal—often cause more excessive removal of cementum and/or dentin than is necessary [11]. Because past studies have documented that biofilm, rather than calculus, is the main culprit in triggering periodontal inflammation [12], other strategies of investigation include methods that eliminate or inactivate the purported periodontal pathogens in the biofilm. As a consequence, systemically and locally applied antibacterial agents (i.e., chemical agents) were used, which notably always bear the risk of bacterial resistance, tolerance, or other side effects [13]. Therefore, alternatives have been introduced to the market to serve as adjuncts during instrumentation in removing or—at least—reducing or modifying bacterial biofilms. The use of lasers and antimicrobial photodynamic therapy (tPDT) has also been the subject of much study recently [14, 15]. While the results of these studies have been inconclusive, the background theory of mechanism remains interesting: selective, light-induced elimination/reduction of microorganisms, with minimal damage to host tissues. As an alternative, but based on mechanical principles, glycine powders using small and soft amino acid particles have been developed for air abrasion, which can be applied in specially designed power jet devices directly on the root surfaces. They have become a real alternative with good clinical and microbiological outcomes and were shown to exhibit less abrasive effects on teeth as compared to hand or ultrasonic scaling or powder jet devices employing classical bicarbonate powder [16–21].

Another chemical line of investigation has recently opened up, with a new gel that was designed to detoxify and clean periodontal pockets. The active ingredients of this gel contain sodium hypochlorite (0.95 %) and amino acids (glutamic acid, leucine, lysine). Based on studies using a similar formulation for the removal of carious dentin lesions [22, 23], this further development now aims to extend the use of this gel mixture for subgingival use by disrupting bacterial biofilms and dissolving degenerated tissues [24]. These effects are purportedly achieved through the chemical reaction of sodium hypochlorite with the amino acids to form N-monochloroamino acids, which while capable of dissolving degenerated tissue, also minimize the detrimental effects of the hypochlorite on sound dentin and healthy soft tissues [25, 26].

Since the spraying of periodontal pockets using a variety of prophylactic powders has recently been introduced as a means to condition tooth root surfaces, little is known regarding its effect on alterations of root surface morphology or the potential cell repopulation thereafter. This also holds true for the application of the buffered hypochlorite gel. Because the regeneration of periodontal tissues relies on a biocompatible dentin surface with minimal surface alterations, the aim of the present study was to examine morphological changes of dentin surfaces following Air-Flow powder or gel application and to assess the influence on PDL cell survival, attachment, and spreading to dentin discs in vitro.

Materials and methods

Dentin disc preparation, cell source, and reagents

Bovine roots of freshly extracted teeth were separated from their crown and the approximate area was first ground flat and polished using water-cooled silicon carbide paper (Stuers, Erkrat, Germany) up to P4000 grit and discs with a diameter of 6.0 mm and a thickness of 1.5–1.6 mm to fit directly into 96-well in vitro culture plates. Dentin discs were prepared using a diamond-coated trephine under constant water-cooling. The discs were then stored in the dark in tap water at a temperature of 4 °C until the experiment started.

Air-Flow® powders (1) Classic, (2) Plus, and (3) Perio were kindly provided by Electro Medical Systems (EMS, Nyon, Switzerland). Perisolv®—composed of hypochlorite (NaOCl) solution buffered with different amino acids—was provided by Regedent (Zurich, Switzerland).

For dentin disc preparations, discs were air sprayed with each powder for 10 s per disc followed by 10 s of rinsing. Perisolv® dentin discs were rinsed with Perisolv® for 10 s followed by rinsing.

Primary human PDL cells were obtained from the middle third portion of three teeth extracted from healthy patients with no signs of periodontal disease extracted for orthodontic reasons as previously described [27, 28]. For ethical approval, informed written consent was obtained from all patients. Primary human PDL cells were detached from the tissue culture plastic using trypsin solution. Cells used for experimental seeding were from passages 4–6. Cells were cultured in a humidified atmosphere at 37 °C

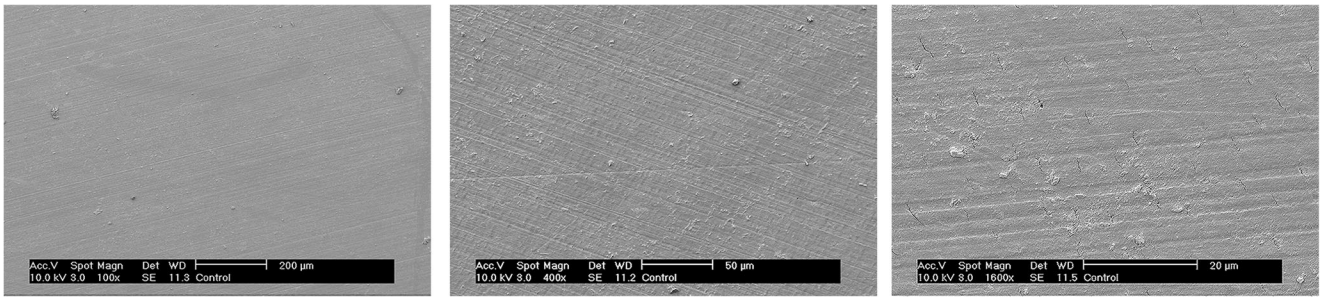

Fig. 1 SEM images of control dentin slices at low (×100), medium (×400), and high (×1600) magnification. Smooth surfaces were observed at low magnifications with slight variations observed at high magnification (×1600)

in growth medium consisting of DMEM (Gibco, Life technologies, Carlsbad, CA), 10 % fetal bovine serum (FBS; Gibco), and 1 % antibiotics (Gibco). For in vitro experiments, cells were seeded with HA in 96-well culture plates at a density of 5000 cells per well for all experiments including cell attachment, cell survival (live/dead assay), and morphological variation as qualitatively assessed via SEM.

Scanning electron microscopy

Dentin discs from samples including (1) control, (2) Powder Classic, (3) Powder Plus, (4) Powder Perio, and (5) Perisolv® rinsing were fixed in 1 % glutaraldehyde and 1 % formaldehyde for 2 days for scanning electron microscopy (SEM). Following serial dehydration with ethanol,

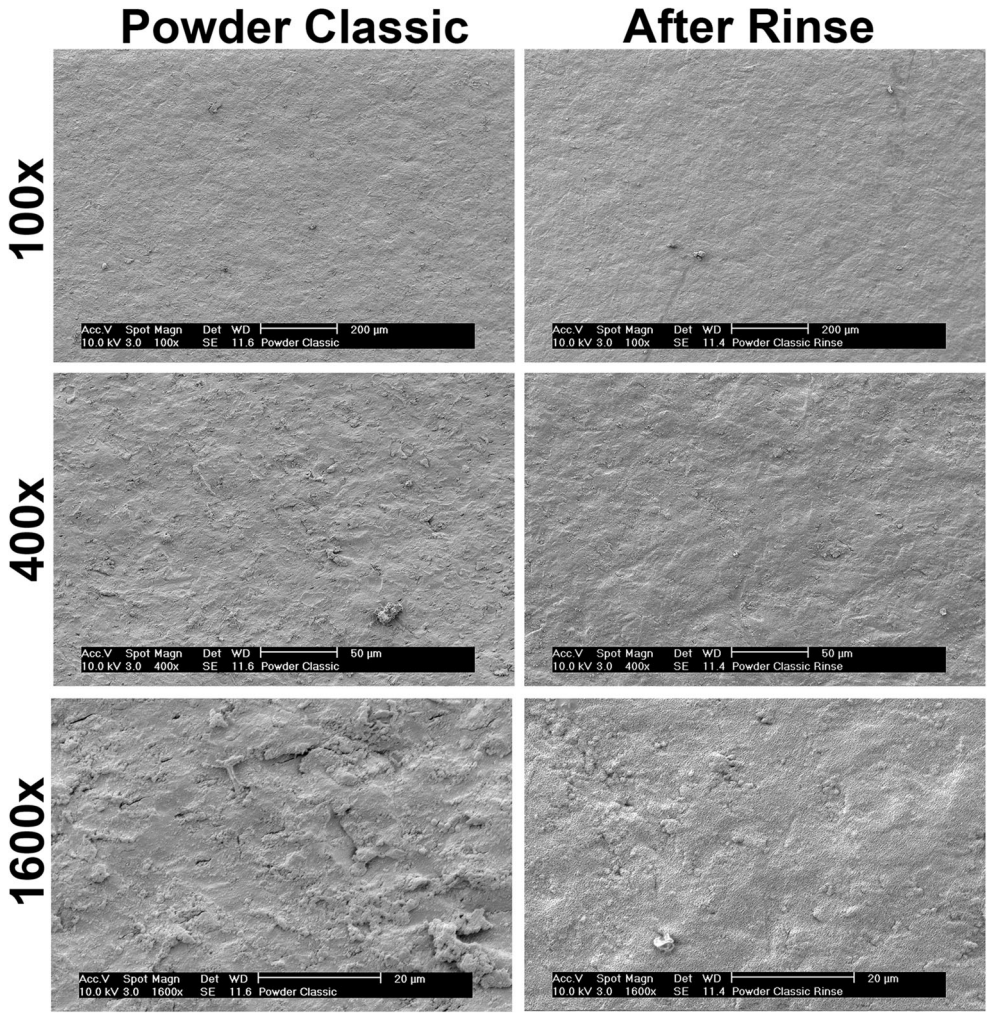

Fig. 2 SEM images of dentin discs Air-Flow sprayed for 10 s with Powder Classic before and after 10 s of rinsing with saline solution at various magnifications. A thin layer of collected powder was observed on dentin discs before and after rinsing

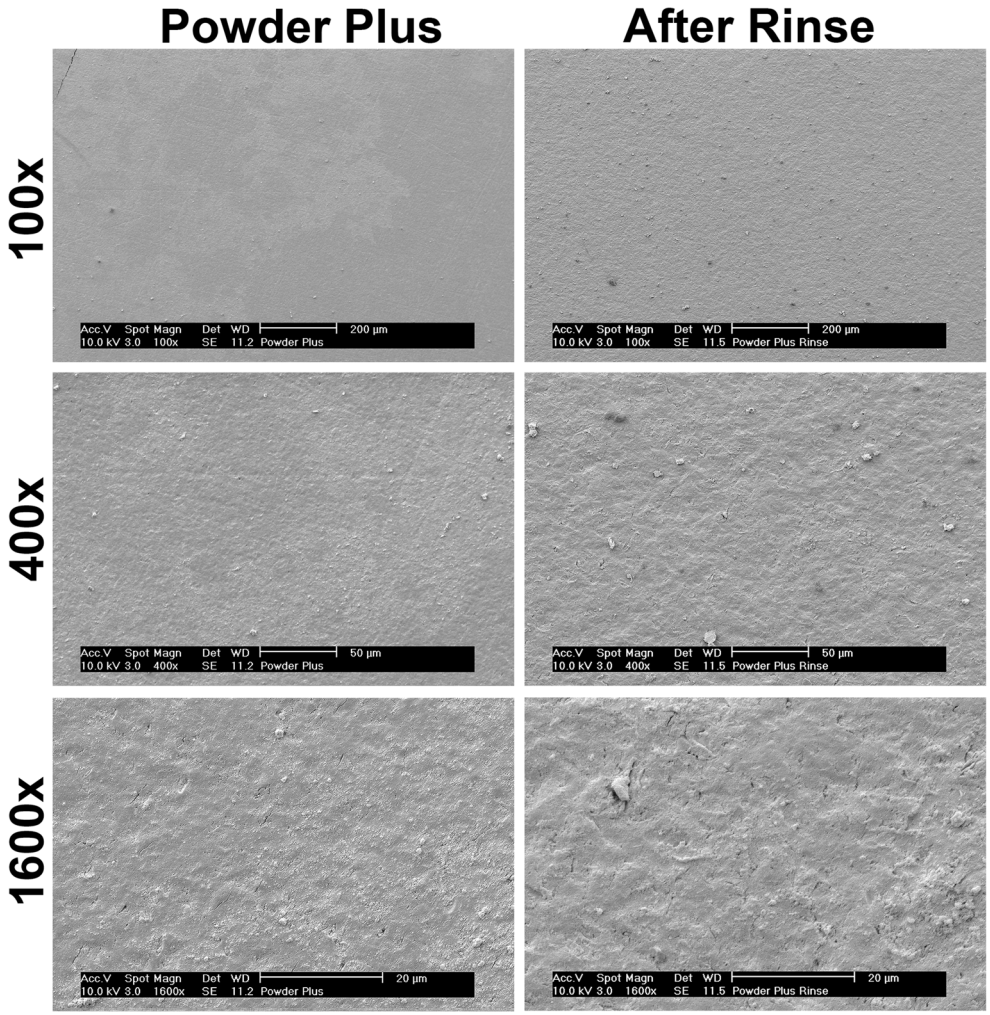

Fig. 3 SEM images of dentin discs Air-Flow sprayed for 10 s with Powder Plus before and after 10 s of rinsing with saline solution at various magnifications. Similarly to Powder Classic, a thin layer of powder was observed on dentin surfaces following spraying

samples were critical point dried (Type M.9202 Critical Point Dryer, Roth & Co. Hatfield, PA, USA) and allowed to dry overnight as previously described [29, 30]. The following day, samples were sputter-coated using a Balzers Union Sputtering Device (DCM-010, Balzers, Liechtenstein) with 10 nm of gold and analyzed microscopically using a Philips XL30 FEG scanning electron microscope to determine surface variations between samples. Furthermore, primary human PDL cells seeded onto dentin discs with each treatment modality were also investigated for PDL cell surface spreading in response to the various Air-Flow powders and Perisolv® rinsing.

Cell viability

Primary human PDL cells were seeded in 96-well plates at a density of 5000 cells per well onto dentin discs including (1) control, (2) Powder Classic, (3) Powder Plus, (4) Powder Perio, and (5) Perisolv®. PDL cells were evaluated using a live-dead staining assay according to the manufacturer's protocol (Enzo Life Sciences AG; Lausen, Switzerland) as previously described [31]. Experiments were performed in triplicate with three fluorescent images taken per experimental condition with a fluorescent microscope (OLYMPUS BX51, Tokyo, Japan).

Adhesion assay

Primary human PDL cells were seeded in 96-well plates at a density of 5000 cells per well onto dentin slices either (1) control, (2) Powder Classic, (3) Powder Plus, (4) Powder Perio, and (5) Perisolv®. PDL cells were quantified using fluorescent imaging (from live/dead assay) at 8 h for cell numbers as previously described [32]. At desired time point of 8 h, cells were washed with phosphate-buffered solution (PBS), fixed with 4 % formaldehyde solution

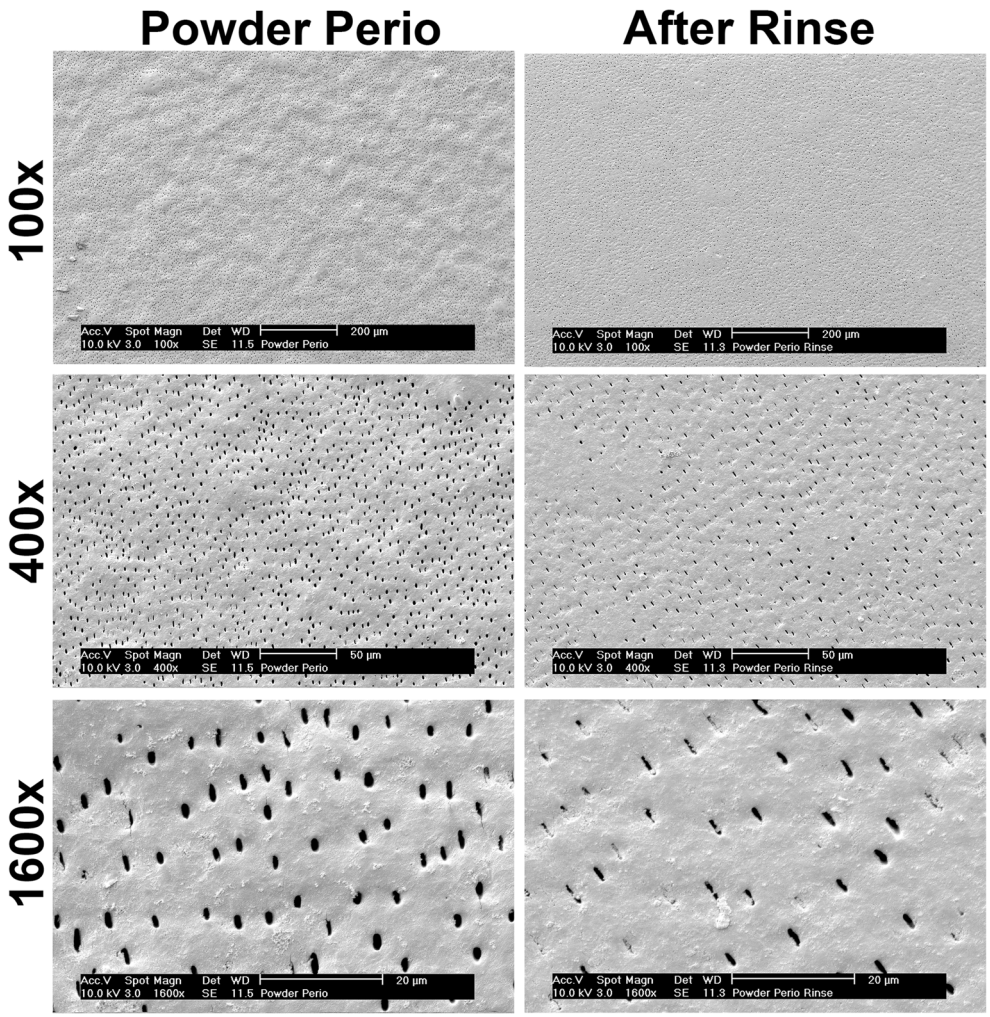

Fig. 4 SEM images of dentin discs Air-Flow sprayed for 10 s with Powder Perio before and after 10 s of rinsing with saline solution at various magnifications. Interestingly, Air-Flow spray with Powder Perio revealed the opening of dentinal tubules both before and after rinsing

(Grogg-Chemie AG, Stettlen, Switzerland) for 5 min, and mounted with VECTASHILD containing DAPI (Vector, Burlingame, CA). Fluorescent images were quantified with a fluorescent microscope. Experiments were performed in triplicate with five images captured per group. Data were analyzed for statistical significance using one-way analysis of variance with Tukey's test (*, p values <0.05 was considered significant).

Results

Surfaces characteristics of dentin slices with or without air polishing or Perisolv® rinsing

Morphological changes to dentin slices were first visualized using SEM imaging (Figs. 1, 2, 3, 4, and 5). First, uncoated control dentin slices demonstrated smooth surfaces at low magnification and demonstrated only slight irregularities at high magnification (Fig. 1). Thereafter, dentin discs were Air-Flow sprayed for 10 s with various powders and visualized before and after rinsing with saline (Figs. 2, 3, and 4). The Classic powder demonstrated the additional layer of powder following Air-Flow, and even after rinsing with saline, fine particles were still observed at high magnification (Fig. 2). A similar observation was observed for Powder Plus however to a lesser extent (Fig. 3). Following rinsing, the dentin surfaces revealed surfaces with many additional micro-rough patterns as a result from the Air-Flow spraying (Fig. 3). Interestingly, dentin discs that were sprayed with Powder Perio demonstrated very profound changes to dentin discs (Fig. 4). It was found that spraying surfaces with Powder Perio revealed the open of dentinal tubules both before and after rinsing (Fig. 4). Lastly, the use of Perisolv® rinsing did not affect surface morphology of dentin discs (Fig. 5).

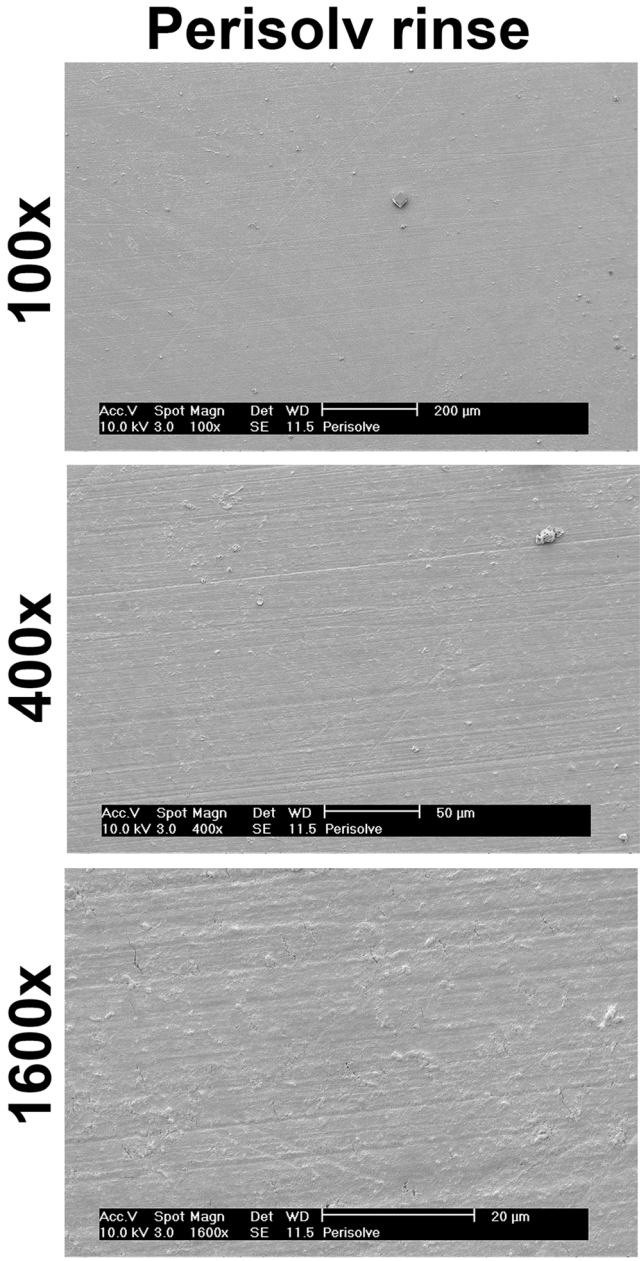

Fig. 5 SEM images of dentin discs that were rinsed with Perisolv® for 10 s at various magnifications. No change in surface morphology was observed when compared to control dentin discs

PDL cell survival, attachment, and spreading

Each of the modifications to dentin discs was then investigated for their effect on PDL cell survival, attachment, and spreading of PDL cells (Figs. 6, 7, and 8). It was first observed that cell survival was near 100 % for all samples (Fig. 6, green cells label live cells versus red cells label dead cells). Thereafter, cell numbers were quantified using DAPI staining at 8 h to investigate the total number of attached cells following each of the treatment groups (Fig. 7). It was found that significantly more cells attached to dentin discs having been Air-Flow sprayed with Perio Powder or rinsed with Perisolv® (Fig. 7). Investigation of cell spreading and cell attachment via SEM imaging did not reveal any discernable differences between treatment groups at 8 h (Fig. 8).

Discussion

Successful periodontal regeneration requires adequate infection control and implies afterwards migration, adhesion, and proliferation of periodontal progenitor and mesenchymal stem cells located in the periodontal ligament [33, 34]. In this context, we focused on biological effects after modern non-destructive root surface cleaning procedures like air polishing or amino acid buffered hypochlorite solution application and determined their influence on PDL cell survival, attachment, and spreading to dentin discs in vitro. This study showed that the Classic and Plus powders demonstrated some coating effects, whereas the Perio powder opened the dentinal tubules even before rinsing. Seeding of PDL cells, however, showed an almost 100 % survival rate on all samples demonstrating very high biocompatibility for all materials despite the smear remnants. Nevertheless, significantly higher cell numbers were observed on samples treated with the Perio powder and the Perisolv® solution, which was corroborated by SEM.

A shortcoming of the present study was that treatments were performed on clean dentin surfaces and that the samples were polished. This comparative screening study, however, primarily focused on material-induced surface changes and the potential influence of the applied materials and their remnants. Therefore, we did not try to imitate the clinical situation in the first instance. Hägi and co-workers assessed air polishing with erythritol with and without chlorhexidine (Plus powder in this study) using a specially designed nozzle for subgingival application and showed that this treatment caused no substance loss and resulted in a smooth surface with nearly no residual biofilm, which also promoted the reattachment of PDL fibroblasts [35]. However, it must be noted that a onefold treatment of the specimens was not sufficient, and that the bacteria had to be additionally killed by UV. And still, the remaining bacterial compounds (e.g., lipopolysaccharides (LPS)) have interfered with PDL fibroblast orientation. In that study, only a fivefold treatment was, however, sufficient to enable a so-called contaminant free and biocompatible surface.

Schwarz and co-workers studied the influence of different air-abrasive powders, glycine, and sodium bicarbonate particles, on cell viability as well [36]. In contrast to the present study, contaminated titanium discs were studied and

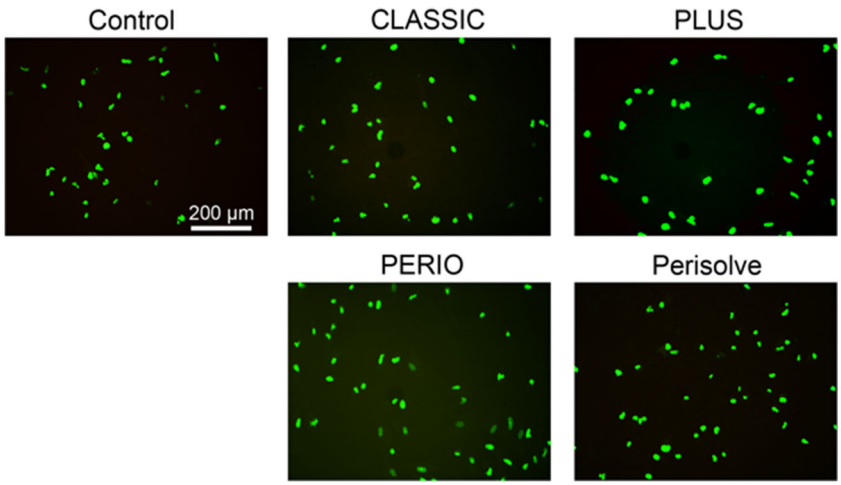

Fig. 6 Live/dead staining of primary human primary PDL cells on control, Powder Classic, Powder Plus, Powder Perio, and Perisolv® dentin discs. For cell viability, live-dead staining was done with viable cell appearing in *green* and dead cells in *red*. The results from these experiments demonstrated that all treatment modalities are highly biocompatible with little to no cell death observed. 13

osteoblastic osteosarcoma cell attachment was measured using a mitochondrial activity assay. Whereas both powders removed almost completely the biofilm, the luminescent cell viability test revealed better cell growth on samples treated with the Classic powder when compared to the Perio powder, which is in contrast to the present study and may be explained at least in part by the different substrates and methods used. The rough titanium surface may have been more efficiently cleaned by the sodium bicarbonate powder, which is characterized by harder particles of a bigger size, which may display an advantage when cleaning this kind of more complex surface structures [36]. On smooth dentin surfaces, in contrast, biofilms are to be removed, whereas the tooth surface preferably remains intact. With bicarbonate powder, considerable substance defects are associated even after short application times of 5 s, whereas glycine powder shows significantly less defect formation. The latter shows no detectable substance loss within the first 5 s [21] and only moderate superficial defects after 20 s of application time. Again, the laboratory condition may differ from the clinical situation in terms that cementum may cover the roots. Both tooth substances differ slightly in their chemical and histologic composition, i.e., that dentin is more mineralized, whereas cementum contains a bigger organic component and more water. This fact should also be taken into consideration when interpreting the current results. Cell attachment may vary as well on cementum. But to obtain samples with intact cementum is (i) difficult, and (ii) we used machined surfaces because this more reflects the clinical reality. However, flat surfaces had to be used under the current laboratory conditions to perform our experiments as planned. In addition, periodontally affected roots were pretreated in most cases. This inflicts partial removal of intact cementum and flattening in due course of the debridement procedures and the root material is abraded in order to ensure a clean and smooth surface. This is necessary—as mentioned above—to obtain a biocompatible surface. But atraumatic surface treatments are still warranted.

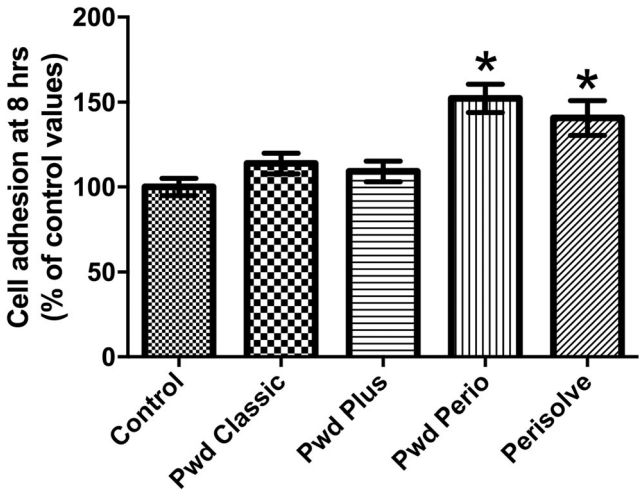

Fig. 7 Cell number of primary human PDL cells seeded on control, Powder Classic, Powder Plus, Powder Perio, and Perisolv® dentin discs. A significant increase in cell numbers was observed on Powder Perio and Perisolv® dentin discs when compared to control samples (*asterisk* denotes significant difference when compared to control samples, $p < 0.05$)

Based on studies using a similar formulation for the removal of carious dentin lesions, this further development of the gel mixture for use subgingivally has been reported in a case study treating 15 patients and a total of 158 residual pockets (non-responding sites persisting beyond the normal healing time of 6–12 months) [22, 23]. The manufacturer's claim is that the gel aids in hard deposit removal (reduced friction during instrumentation, softening of calculus), disruption of biofilm, and dissolving the generated tissue and therefore facilitating its removal from the periodontal pocket by scaling and root planning and aids in the healing process through its antibacterial properties [24].

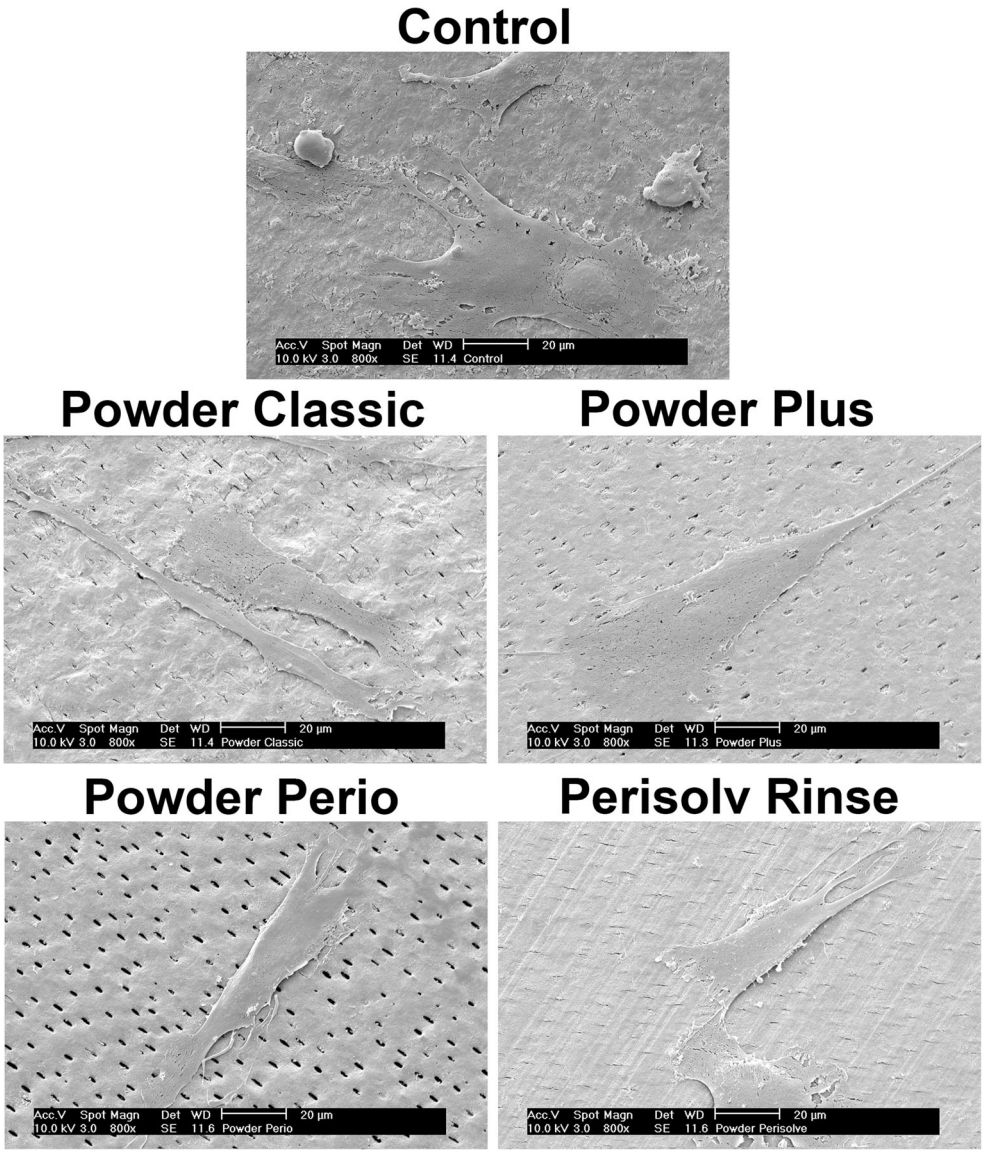

Fig. 8 SEM images of primary human PDL cells seeded on control, Powder Classic, Powder Plus, Powder Perio, and Perisolv® dentin discs. No discernable differences could be observed with respect to cell shape or spreading following surface modifications between groups

Therefore, no harmful side effects have been reported in over its 15-year use for caries removal, and none are to be expected with its use in the treatment of periodontal pockets. However, laboratory or clinical data regarding the latter indication are still scarce. Therefore, this study was justified and the results corroborated some assumptions within the limitations of the present investigation. One other reported limitation of the present study was the time course investigation culturing primary human PDL cells onto dentin surfaces. While we report that all treatment modalities were able to re-establish periodontal cell repopulation, future investigation with longer time points is of interest to further determine the ability for each treatment modality to influence PDL cell proliferation and mineralization. Furthermore, numerous cell types are in contact with dentin/cementum surfaces including gingival fibroblasts and epithelial cells. Future research investigating the various cell types found in contact with dentin and cementum surfaces are needed to evaluate the potential of each air polishing or amino acid buffered hypochlorite solution technique on cell behavior of gingival fibroblasts and epithelial cells.

In summary, the present study demonstrated that cell survival and repopulation of root surfaces is possible following either air polishing or application with Perisolv®. Additional in vitro and animal testing is necessary to further characterize the beneficial effects of either system in clinical setting. Potential side effects when applying these techniques and materials should also be taken into consideration, when it comes to the opening of dentinal tubules and related consequences, especially when treating sensitive areas and patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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Ethical approval This article does not contain any studies with identifiable human participants or animals. An IRB was therefore not required.

Informed consent For this type of study, signed informed consent was obtained for PDL cell isolation.

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Basic evaluation of an antimicrobial gel for peri-implantitis treatment

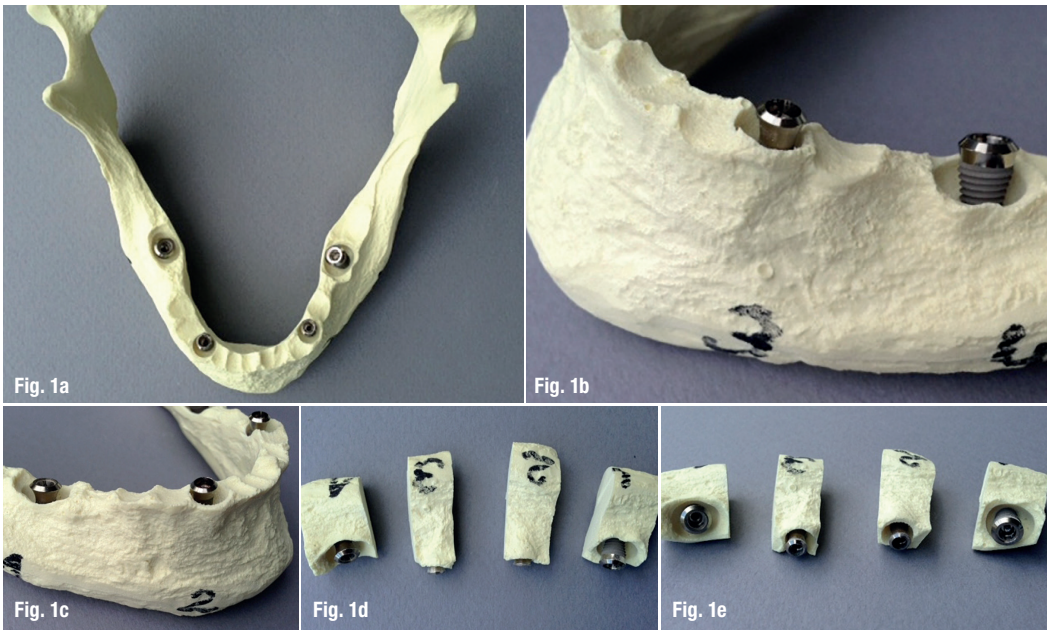
Authors: Dr Georg Bach & Christian Müller, Germany

Introduction

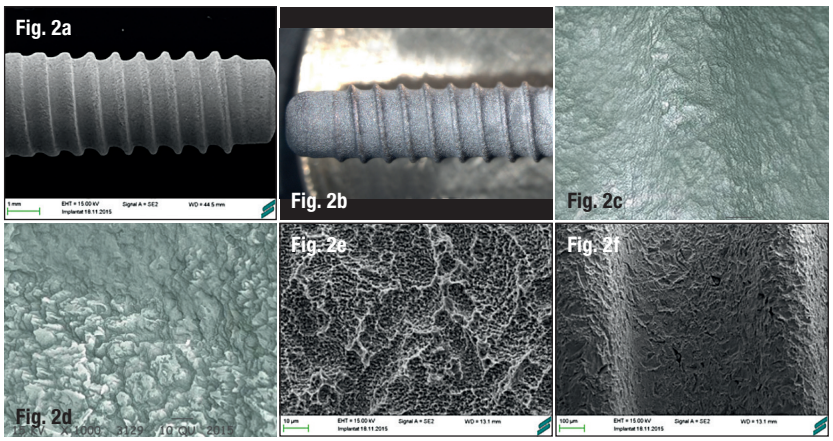
Early complications, which have been regarded as the major dread in the initial phase of oral implantology, have become a rare phenomenon for a fairly long time. Reasons for this positive development can be found in significant improvements of the implant surfaces, improved insertion techniques as well as in new ways to improve the prospective implant site.

Nevertheless, with the enormously increased number of inserted implants, a significant increase of late complications has meanwhile been recorded.[1,4,12] These complications typically manifest themselves many years after installation of the superstructure by means of peri-implant bone loss around artificial tooth pillars.[17,20,21,25] Often associated with an insufficient or declining oral hygiene of the patient, these peri-implant lesions lead to the loss of the artificial tooth pillar and the corresponding suprastructure in case they are not treated.[5,11,13,14] Many authors regard the development of peri-implantitis therapies as one of the current key challenges of implantology.[15,18-20,26]

Cleaning and disinfection of the exposed implant areas represents an undeniable requirement. For the latter step the term "decontamination" has been generally established.[3,16] For decontamination, various methods are indicated for their suitability.[3,6,8,16,21-24] The aim of this study was to evaluate the suitability of using an antimicrobial gel for peri-implantitis treatment in an *in-vitro* experiment.



Figs. 1a–e: Peri-implant defect—Simulated model: Crater-shaped defects were prepared in plastic jaws typically used for insertion exercises. Brand-new implants were placed in the middle of these defects in a way that at least three threads were exposed **(a–c)**. The jaws were divided into smaller units **(d & e)** and autoclaved before conducting phase II examinations (bacterial cultivation —Perisolv application—Microbiological diagnostics etc.) in order to allow better fit into the furnace as well as in vials containing culture medium.



Figs. 2a–f: SEM analysis: Brand-new, sterile implants were inoculated and incubated with a microbial suspension. **Figure 2a** shows a scanning electron micrograph of this starting material. **Figure 2b** shows the bacterial turf on an implant thus processed. After Perisolv application, many areas showed a detached bacterial coating, the implant surface is virtually free from bacterial turf **(c & d)**. These "exposed spots" feature an unchanged implant structure **(e & f)**, therefore Perisolv application does not alter the implant surface per se.

Material and Methods

Two test phases were performed:

a) Phase I: Decontamination procedure of brand-new sterile implants, which have been inoculated with bacteria and subsequently coated with antimicrobial gel.

b) Phase II: Decontamination procedure of brand-new sterile implants placed in a plastic jaw with simulated bone defects after subsequent inoculation with bacteria and final exposure to antimicrobial gel.

Phase I: Decontamination procedure to implants inoculated with bacteria

To evaluate general suitability of the decontamination process, brand-new ITI implants (Institut Straumann AG, Basel, Switzerland) were microbiologically processed and analysed at the Institute for Medical Diagnostics Bioscientia (Freiburg, Germany).

Implant contamination—microbial procedure:

The implants were exposed and inoculated with a bacterial suspension (overnight cultures of *MRSA ATCC 33591*):

By means of sterile forceps, the implants were placed in 10 ml peptone yeast extract broth each. The tubes were incubated for 48 h at 36 °C and 5–10 % CO_2. After 48 h of incubation, the liquid was removed by means of vacuum filtration and the implant was transferred back to the initial container with sterile forceps for immediate further processing. Exclusively, implants with a medium bacterial growth were used for further examinations, implants with low or very low bacterial growth were excluded. Two test series were conducted with four implants each.

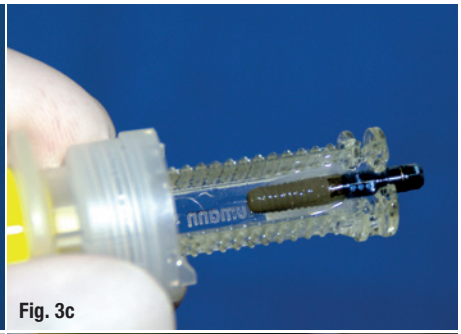
Decontamination procedure with contaminated whole implant bodies:

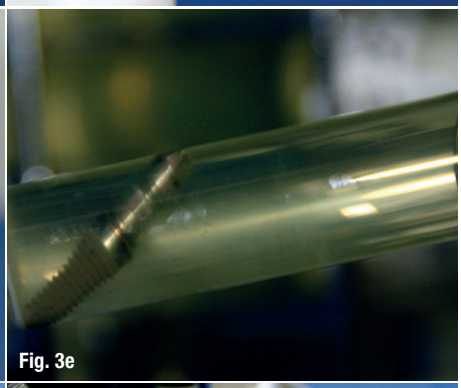
After completion of the microbiological work, three out of four implants were confronted with antimicrobial gel for two min in the sense of a decontamination procedure and immediately transferred to the Institute for microbiological analysis. One implant served as positive control, without conduction of the decontamination procedure.

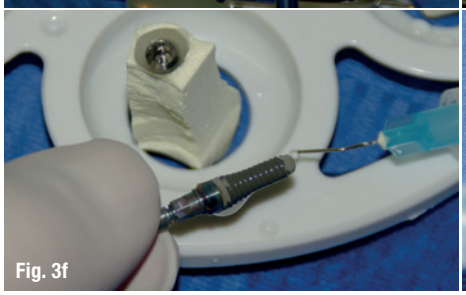
- Antimicrobial Gel: An antimicrobial gel known for its application in periodontology was used (PERISOLV, REGEDENT AG, Zurich, Switzerland). It is typically used for adjuvant cleaning and decontamination of the outer tooth root area and the surrounding tissue.[10] Furthermore, in the literature the gel is described to feature a softening effect towards degenerative tissue before debridement of periodontal pockets.[9] According to the manufacturer, the gel does not affect healthy tissue[9] and, however, features an antimicrobial effect.[2,7]
- Gel composition: The gel contains amino acids (glutamic acid, leucine and lysine), carboxymethyl cellulose, titanium dioxide as well as ultra pure water and features a pH value below 10. The transparent liquid represents a 0.95 % sodium hypochlorite solution and is admixed immediately before the application. After mixing hypochlorite and amino acids, so-called Chloramines (NCA), a short-lived active substance class, are formed. These substances are part of the body's own immune system.[9]
- Gel Preparation: The set (gel and liquid) is stored in the refrigerator. One hour prior to planned application, the set is removed from the refrigerator to allow the contents of the kit to warm up to room temperature. Both components (gel and liquid) are arranged in separate syringes and are connected by means of screwing (Luer-lock connection). Both components were thoroughly mixed by moving the stamps back and forth 10–15 times. The activated and operational gel was finally left in the transparent syringe. A non-invasive/blunt application tip is attached and the implants are coated with the gel.

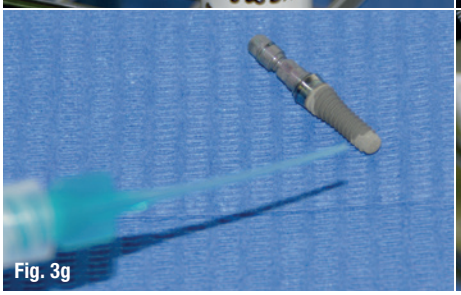
Table 1: Results of Phase I.

	Bacterial growth on implant	Implant 1	Implant 2	Implant 3	Implant 4 control
A:	MRSA	–	–	–	+ + +
B:	MRSA	–	+	–	+ + +









Figs. 3a–h: Phase I: Brand-new, sterile implants were used for the study. Implants supposed for SEM evaluation were initially kept in their original containers. The MRSA bacterial suspension was drawn in a sterile, disposable syringe **(a)** and applied directly on the respective implant in its original container **(b & c)**. Subsequently, the shipment for immediate SEM analysis was carried out. Implants supposed for microbiological testing were removed from their containers and placed directly into the MRSA bacterial suspension **(d & e)**. After a one-minute inoculation period, the implants were removed and coated with Perisolv gel **(f & g)**. After the exposure time specified by the manufacturer, the implants were introduced into the tube containing the nutrient medium and sent to the microbiological examination **(h)**.

Implant preparation for microbial investigations

Immediately after application of the gel, the implants were introduced into tubes with a sterile nutrient solution and sent to the Institute for microbiological analysis. The samples were processed in the Microbiological Institute by means of conventional (plate) cultivation.

Scanning electron microscopic studies of the implants

Some of the implants were investigated by scanning electron microscopy (Institut Straumann AG).

Results of Phase I—Decontamination procedure with contaminated whole implant bodies (Tab. 1)

Scanning electron microscopic studies

In some areas, where Perisolv had been applied, the "bacterial turf" on the implants was interrupted or rather dissolved/removed. Underlying areas, freed from bacterial turf, displayed an intact, unaltered implant structure. For implants only confronted with Perisolv without previous inoculation, no gel-induced change of the implant surface were observed.

In summary, SEM analysis after treatment with the gel revealed no change of implant surface as and a partial dissolution of the inoculated bacterial layer.

Microbiology

Phase I investigations revealed bacterial inactivation in the highest degree, remaining MRSA bacteria were detected in one test item of series B1 only.

Summary of Phase I—Decontamination procedure with contaminated whole implant bodies

The investigated gel is capable to induce a pronounced destruction of pathological bacteria present on implant surfaces without altering this implant surface structure.

Phase II: Testing the effect of the antimicrobial gel on contaminated implants placed in a plastic jaw with a simulated peri-implant tissue defect

After the first test phase to evaluate the principle suitability of the gel application, a second test phase was conducted.

Table 2: Results of Phase II.

	Bacterial DNA in simulated bone defect	Unit 1	Unit 2	Unit 3	Unit 4 Control
A:	MRSA	++	+	++	+++
B:	MRSA	–	++	+	+++

Preparation of simulated peri-implant defects

Implants (Institut Straumann AG) were placed in a plastic jaw, which was prepared with standardised crater-shaped (peri-implant) defects prior to implant placement. The implants were placed in the centre of these defects by means of allowing the upper three threads not to be sunk into the plastic. Thus, a defect situation simulating a typical manifested peri-implantitis was generated. For better further processing, the jaws were sawed into small implant/plastic jaw units. These implant/plastic jaw units were steam sterilised (autoclaved).

Implant contamination

Afterwards, the exposed implant surfaces were contaminated with a bacterial suspension. The circumferential defects were completely filled with the bacterial suspension as well. Two test series were conducted with four implant/plastic jaw units each.

Microbiological procedure:

The bacterial suspension (*MRSA ATCC 33591*–ATCP strain) was prepared and suspended in BHI broth. The bacterial count of this "stock suspension" represented approx. 10^8–10^9 bacteria/mL. To inoculate the implant/plastic jaw units, each 100 µl of the cultured MRSA stock suspension were pipetted into one simulated bone defect. This corresponds to approx. 10^7–10^8 bacteria/100 µl respectively.

Decontamination procedure with simulated peri-implant defects

Perisolv gel was administered into three of four simulated bone defects (details s. Chapter "Phase I"). The gel was allowed to operate for two minutes. One implant/plastic jaw unit served as a positive control, where no decontamination was performed.

Implant preparation for microbial investigations

The units were subsequently placed into 10 mL of BHI broth (Brain Heart Infusion Glucose), each by means of a sterile forceps. The implant/plastic jaw units were placed in a culture oven. To establish a humid environment, a small Erlenmeyer flask filled with sterile distilled water was added into the pot. The units were incubated under aerobic conditions at 36 °C.

After two days of incubation, the simulated bone defect of unit 1 was dry, whereas bone defects of units 2–6 were still slightly humid. The remaining liquid from these units was removed by means of a pipet.

The implant/plastic jaw units were introduced in tubes with a sterile nutrient solution and forwarded to the Institute Bioscentia for microbiological analysis. The samples were processed by means of conventional (plate) cultivation.

Results of Phase II (table 2)

Remaining MRSA bacteria were detected in five of six decontaminated implant/plastic jaw units as well as in the control unit. This finding can be categorised as "significant" in three out of five units and as "distinct" in the other two out of five units. In addition, a bacillus species was detected in one unit. This can be regarded as an environmental contaminant.

Figs. 4a–i: Phase II: Brand-new, sterile implants were placed in simulated bone defects in a plastic jaw. These implant/plastic jaw units were autoclaved. Afterwards, a MRSA solution was introduced into the simulated peri-implantitis defects **(a–c)**. Afterwards, the units were incubated in a special oven and a proof for the presence of "massive" MRSA bacteria was performed. At this time, the Perisolv gel was applied **(d–f)**. After the exposure time specified by the manufacturer, the samples were placed directly into a BHI broth **(g & h)** and the samples were passed for further microbiological examination **(i)**.

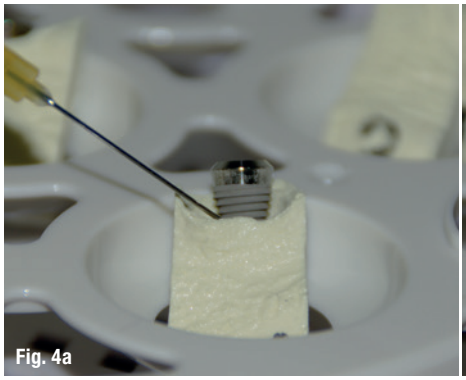


Fig. 4a

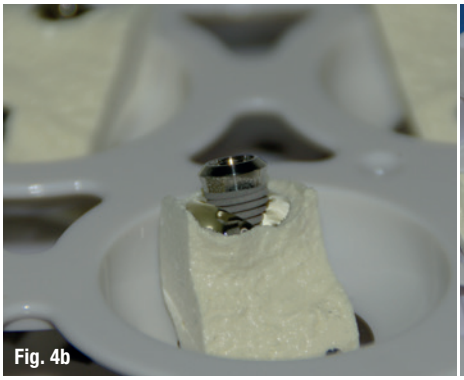


Fig. 4b

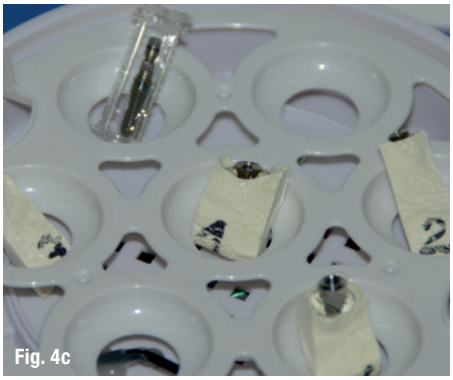


Fig. 4c

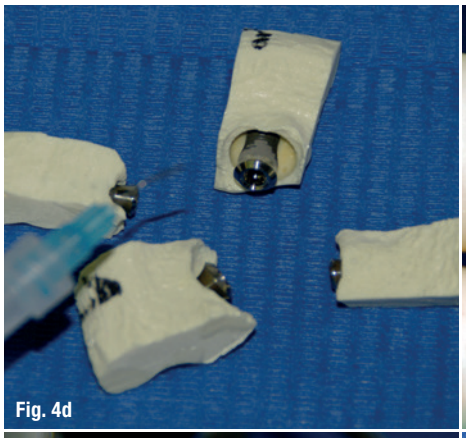


Fig. 4d

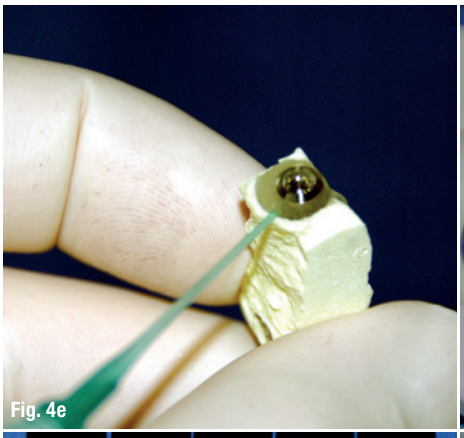


Fig. 4e

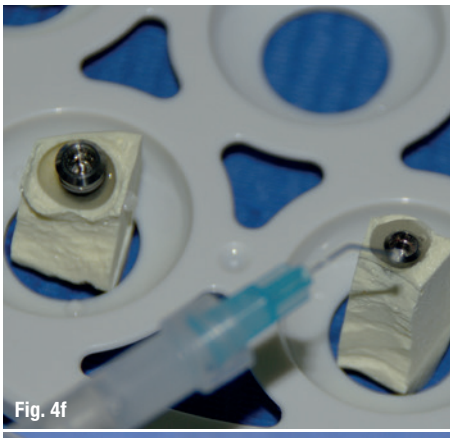


Fig. 4f

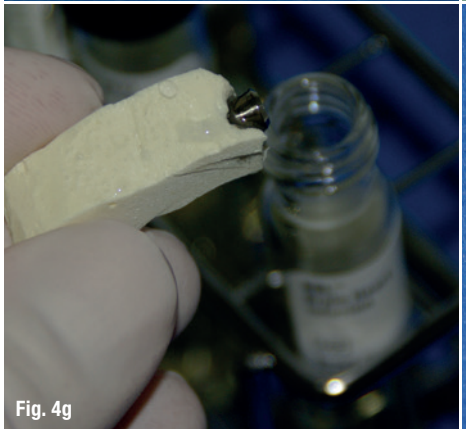


Fig. 4g


Fig. 4h

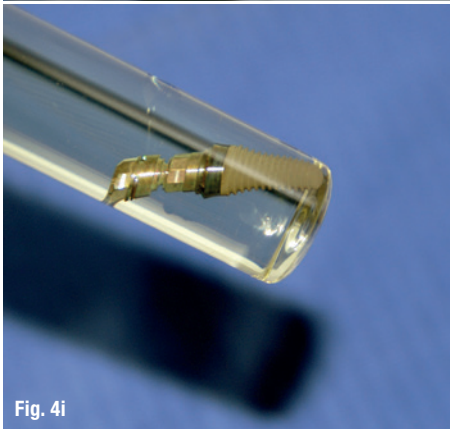


Fig. 4i

Breeding trial after decontamination

It was possible to recultivate bacteria sporadically after decontamination and simple drying.

Preliminary Summary

Compared to other decontamination procedures, the application of the antimicrobial gel Perisolv achieved satisfactory decontamination results from a microbiological point of view in both *in-vitro* study phases. In all samples, a significant reduction of the bacterial count was observed. However, a bacterial elimination only was achieved in the first study phase, but not in the second phase.

SEM images of the implants that have undergone the procedure described above, pointed out that the antimicrobial gel did not induce any changes to the implant surface and that it has certain potency for dissolving the (inoculated) bacterial turf.

As a limitation to the evaluated results, it should be clearly stated that the presented investigation was performed in an *in-vitro* environment with a "non-human milieu" and without a real inflammatory component. Thus, our results about the basic applicability of the presented method can be regarded as a first approach, but in no case a clear statement about the definitive decontamination efficacy of the tested methods can be done.

Acknowledgement

In particular, we want to thank Dr Brodner (Institute Bioscientia, Freiburg, Germany) and Institut Straumann AG (Basel, Switzerland) for their valuable support in the microbiological testing phase and in the preparation of scanning electron images. We want to thank Straumann Germany GmbH for providing the plastic jaws and the implants. Without their elaborate and valuable work, this study would not have been possible._

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ORIGINAL ARTICLE

In-vitro activity of sodium-hypochlorite gel on bacteria associated with periodontitis

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Abstract
Objectives The aim of the present study was to assess the antimicrobial activity of a sodium hypochlorite formulation including its components against bacteria associated with periodontal disease.
Materials and methods Sodium hypochlorite formulation (NaOCl gel), its components sodium hypochlorite (NaOCl), and the activating vehicle were compared with 0.1 % chlorhexidine digluconate (CHX) solution. The antimicrobial activity was proven by determination of minimal inhibitory concentrations (MIC), minimal bactericidal concentrations, and killing assays. Furthermore, the influence on formation as well as on a 4-day-old 6-species biofilm was tested.
Results Except for one strain (*Parvimonas micra* ATCC 33270 in case of NaOCl gel), the MICs both of the CHX solution and NaOCl gel did not exceed 10 % of the formulations' concentration. In general, MICs of the NaOCl gel were equal as of the CHX solution against Gram-negatives but higher against Gram-positive bacteria. CHX but not NaOCl gel clearly inhibited biofilm formation; however, the activity of NaOCl gel was more remarkable on a 4-day-old biofilm. NaOCl killed bacteria in the biofilm and interfered with the matrix.
Conclusions The NaOCl gel acts antimicrobial in particular against Gram-negative species associated with periodontitis. Moreover, its component NaOCl hypochlorite is able to alter biofilm matrices.
Clinical relevance The NaOCl gel may represent a potential alternative for adjunctive topical antimicrobial treatment in periodontitis.

Keywords Sodium hypochlorite · Periodontal infection · Biofilm

Introduction

Periodontitis is a chronic inflammatory disease of the tooth supporting tissues associated with high counts of certain bacterial species interacting with the host' immune system [1]. Oral microbial-plaque communities are biofilms composed of numerous bacteria on host surfaces. [2]. Bacteria more present in patients with chronic periodontitis than in periodontally healthy subjects are *Treponema denticola*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and several others [3]. *P. gingivalis* a Gram-negative anaerobe bacterium is considered a key-stone pathogen in developing periodontal disease [4].

Non-surgical mechanical removal of the hard and soft microbial deposits from the root surfaces (i.e., scaling and root planing (SRP)) is the standard in any cause-related periodontal therapy [5]. Substantial evidence indicates that during supportive periodontal therapy (SPT), periodontitis can be successfully treated and controlled by thorough mechanical plaque removal by the patient coupled with supra-and subgingival debridement by the therapist with or without the

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use of local antimicrobials [6]. During the last decade, various antimicrobials such as chlorhexidine, azithromycin, metronidazole, doxycycline, minocycline, and tetracycline used subgingivally in conjunction with SRP have been tested [7]. Among the supragingivally used antiseptics, chlorhexidine-based dentifrices/gels are still being the gold standard although tooth surface discoloration is observed as side effect [8].

An alternative approach to improve the outcomes of subgingival SRP might be the application of sodium hypochlorite. Its broad antimicrobial activity, fast bactericidal action, and non-toxicity at application concentration, has been known since many years [9]. Already in 1918, it was published that sodium hypochlorite has a higher dissolving effect on necrotic tissue than on vital one [10]. However its use on everyday basis in dentistry is known basically in endodontics as one of the main canal irrigants [11]. Activity of sodium hypochlorite is depending on pH. It is most active when applied at neutral or slightly acidic pH [12]. Comparison of the activity of different antiseptics on experimental biofilm with various endodontic/periodontal pathogens showed that the highest bactericidal activity was obtained with 2.25 % sodium hypochlorite and 10 % povidone-iodine and last by 0.2 % chlorhexidine [13]. Already in the early 80s of the last century, the use of sodium hypochlorite combined with curretage was histologically shown to be effective in reducing soft tissue inflammation in SPT [14]. The results showed that sodium hypochlorite achieves predictable chemolysis of the soft tissue wall of the periodontal pocket with minimal effect on the adjacent tissues while the antiseptic did not impede the healing phase [14]. Moreover, it was suggested by Perova [15], that the use of 0.1 % sodium hypochlorite during periodontal surgeries might improve the healing, through a markedly better regeneration of the connective tissue at the gingival base of the sites. Despite its promising properties, sodium hypochlorite did not stay in the light of interest for long until it has been rediscovered just recently. An oral mouth rinse with 0.05 % sodium hypochlorite resulted in significant reduction in supragingival biofilm accumulation and gingival inflammation [16]. In subsequent studies, twice-weekly rinsing with 0.25 % sodium hypochlorite solution decreased dental plaque level and reduced bleeding on probing in periodontal pockets [17, 18].

A formulation of a sodium hypochlorite gel to be used in periodontal therapy has been recently introduced to the market. It is composed of two components, sodium hypochlorite solution and a mixture of amino acids. After combining the two substrates, different chloramines are synthesized from free sodium hypochlorite, which may additionally enhance the antimicrobial activity.

The purpose of this in vitro study was to determine the antimicrobial activity of the sodium hypochlorite gel and its components in comparison to chlorhexidine digluconate on microbial species associated with periodontitis including *P. gingivalis*, *T. forsythia*, *Aggregatibacter actinomycetemcomitans*, *Parvimonas micra*, and others.

The hypothesis was that there is a minimal inhibitory concentration of sodium hypochlorite gel, which inhibits predictably the growth and biofilm formation of bacteria associated with periodontal disease.

Material and methods

Substances

Test substances were component 1 (NaOCl: sodium hypochlorite solution 0.95 %), component 2 (activating vehicle: glutamic acid, leucine, lysine, carboxymethyl cellulose, and ultrapure water), and sodium hypochlorite gel (NaOCl gel (Perisolv, Regedent AG, Zurich, Switzerland)), composed of the previous two components mixed together. Chlorhexidine digluconate solution (CHX) in the concentration of 0.1 % was used as a positive control, whereas 0.9 % sodium chloride (NaCl) as a negative control.

Microorganisms

The following bacterial strains were tested as single bacterial species: *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037, *Fusobacterium nucleatum* ATCC 25586, *Streptococcus gordonii* ATCC 10558, *Actinomyces naeslundii* ATCC 12104, *Parvimonas micra* ATCC 33270, *Prevotella intermedia* ATCC 25611, *A. actinomycetemcomitans* ATCC 33384, *Campylobacter rectus* ATCC 33238, *Eikenella corrodens* ATCC 23834, *Filifactor alocis* ATCC 33099, *Capnocytophaga gingivalis* ATCC 33624, *Eubacterium nodatum* ATCC 33270, and three clinical isolates of *P. gingivalis* and *T. forsythia*. The mixed microbiota consisted of the following bacterial strains: *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037, *F. nucleatum* ATCC 25586, *S. gordonii* ATCC 10558, *A. naeslundii* ATCC 12104, and *P. micra* ATCC 33270. Before an experiment, all strains were precultivated on Schaedler agar plates (Oxoid, Basingstoke, UK) with 5 % sheep blood and vitamin K addition, in an anaerobic atmosphere or with 5 % CO_2 (*A. actinomycetemcomitans* ATCC 33384 and *S. gordonii* ATCC 10558).

Susceptibility tests: determination of the minimal inhibitory concentrations and minimal bactericidal concentrations

Determination of MICs was performed by the micro-broth dilution technique using the 96-well microtiter plates. The MICs of component 1, component 2, Perisolv, and 0.1 %

chlorhexidine solution for single microorganisms and mixed species were checked.

After subcultivation of bacterial strains, a defined inoculum with an adjusted turbidity of McFarland 4 (0.5 for *S. gordonii*) was added to Wilkins Chalgren broth (Oxoid) supplemented with nicotinamide adenine dinucleotide and N-acetyl muramic acid, in a 1:17 ratio. Defined concentrations of NaOCl gel, its components, and chlorhexidine as positive control were added. After 42 h of incubation time (18 h for *S. gordonii*), the growth of microorganisms was analyzed visually by checking the turbidity. MIC represents the lowest concentration without visible turbidity of the broth.

For determination of the minimal bactericidal concentration, non-turbid cultures were subcultivated on agar plates without the addition of any antimicrobial agent. After incubating, the MBC was the lowest concentration without any growth of the colonies on the agar plates (equivalent to a reduction by 99.9 % of the initial inoculum).

Tests were performed in independent replicates.

Killing

A defined inoculum of microorganisms (about 10^6 /ml), prepared in doubled concentrated nutrient media (Wilkins Chalgren broth), was added to NaOCl gel in final concentrations of 20, 10, and 5 %, as well as 0.01 % chlorhexidine. In this experiment, the following bacterial species were used: *P. gingivalis* ATCC 3327, *T. forsythia* ATCC 43037, *P. micra* ATCC 33270, and the mixed species. After 1, 2, 6, as well as 24 h of incubation, the numbers of viable bacteria were determined by enumeration of colony-forming units (cfu). The test was performed in independent replicates.

Activity against bacteria in biofilms

In these experiments, a multispecies biofilm consisting of *S. gordonii* ATCC 10558, *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037, *F. nucleatum* ATCC 25586, *A. naeslundii* ATCC 12104, and *P. micra* ATCC 33270 was used. The influence of the test substances on the developing biofilm was examined. First, the wells of 96-well plates were covered with 20 μl of test substance. After 1 h of incubation, 10 μl of protein solution consisting of 25 % serum and 5 % albumin was added to the surface, followed by 170 μl of bacterial suspension. Bacterial suspension was prepared by adding a defined inoculum (1 part *S. gordonii*, 2 parts *A. naeslundii* and each 4 parts of the other species, each McFarland 4) to Wilkins Chalgren broth (Oxoid) supplemented with nicotinamide adenine dinucleotide and N-acetyl muramic acid, in a 1:17 ratio. The 96-well plates were incubated anaerobically at 37° under static conditions. The cfu were counted after 6, 24, and 48 h development of biofilm.

Additionally, the influence of the test substances was evaluated on a formed biofilm. Multispecies biofilm composed of six bacterial species was developed for 4 days. First, the wells of 96-well-plates were covered with 10 μl of 25 % *v/v* inactivated human serum/well for 1 h. Then, bacterial suspension was prepared and mixed with the Wilkins Chalgren broth as described above. The plates were incubated in the anaerobic atmosphere at 37 °C. After 48 h, the medium was carefully exchanged with a new bacterial suspension of *P. gingivalis* ATCC 33277 and *T. forsythia* ATCC 43037 added to the nutrient medium before application to the wells. The renewed addition of selected bacterial strains guaranteed a sufficient number of these species within the biofilms.

After an additional incubation for 24 h, the medium was removed carefully, and 20 μl of the tested substances were added to the biofilm. After 1 min, 180 μl of Wilkins Chalgren broth was added, and the plates were incubated for 18 h. Finally, after short washing, the biofilm was carefully scraped, mixed by pipetting, and cfu were enumerated after serial dilutions, spreading of each 25 μl on agar plates and incubation for 7 days.

Half of the 96-well plate was used for staining of the biofilm with the Kwasny and Opperman method [19]. After a manual plate washing, the biofilm was fixed by incubating the washed plate at 60 °C for 60 min. Finally, the staining was performed with 0.06 % (*w/v*) solution of crystal violet dissolved in dH_2O. Fifty microliters of the above stain was used per well and left for 5 min. After the staining procedure, the excess was removed by repeated washing. The amount of crystal violet bounded in each well was directly measured spectrophotometrically by measuring OD_{600} using microplate reader.

These experiments were made in two independent experiments in independent sextuplicates.

In addition, 4-day-old biofilm samples on glass slides treated with the test substances and processed as described above were stained with 0.1 % acridine orange solution (Merck, Darmstadt, Germany) as a general nucleic acid stain. Samples were examined by using fluorescent microscope (Olympus BX51, Tokyo, Japan).

Furthermore, scanning electron microscope photographs were taken to visualize the results. Exemplarily, each test substance was chosen. Samples were fixed in 2 % glutaraldehyde in cacodylate buffer for 30 min, washed twice with cacodylate buffer, and dehydrated using a graded ethanol series (15 min each concentration). Following critical point drying, samples were sputter-coated with gold and examined with a ZEISS LEO-1530 Gemini (Carl Zeiss NTS GmbH) equipped with a field emission electron gun at 8 keV.

Statistical analysis

All data are presented as mean and standard deviation (SD). Data were compared using a one-way analysis of variance

(ANOVA) with post-hoc comparisons of groups using LSD corrections. A p value of 0.05 was considered to be statistically significant. However, in case of log10 cfu values, the cfu log10 reductions are of importance, following only log10 cfu values are presented. SPSS software (version 22.0) was used for statistical analysis.

Results

All results were related in percent to the working (commercially available) concentration (NaOCl gel and 0.1 % CHX, respectively).

Minimal inhibitory concentrations and minimal bactericidal concentrations

Except for one strain (*Parvimonas micra* ATCC 33270 in case of NaOCl gel), the MICs both of the CHX solution and NaOCl gel did not exceed 10 % of the formulations' concentration. In general, MICs of CHX were lower than those of the NaOCl gel. However, when differentiating between Gram-positives and Gram-negatives, the difference was mainly due to the Gram-positive bacteria. NaOCl gel acted more growth inhibitory on Gram-negatives than on Gram-positives. Mainly, NaOCl was the active compound of NaOCl gel. But the activating vehicle exerted also some activities on Gram-negatives; here, a synergistic effect can be stated when comparing NaOCl gel with its compounds (Fig. 1).

The MBC values were in general equal or one step higher than the corresponding MIC values. The difference was more or equal two steps for CHX against two microorganisms (incl. the mixture) and for NaOCl gel against seven microorganisms (incl. the mixture). MBC values and individual MIC data are presented in Supplementary Table 1.

Killing curves

Killing curves show the fast and total killing activity of 20 % of the CHX solution and of the NaOCl gel. Only a few cells of *T. forsythia* were able to survive the exposure to the compounds. NaOCl gel was also tested in the lower concentrations of 5 and 10 %. In part, a concentration-dependent activity was visible (Fig. 2).

Influence on formation of biofilm

A clear inhibition of biofilm formation by CHX is shown. Up to 6 h, no bacteria were cultivable; after 24 and 48 h, less than 0.5 log10 cfu in mean were counted. The effect of NaOCl gel and its compounds was limited. The reduction was equally about 1.4 log10 cfu in mean both for the NaOCl gel as for NaOCl and the activating vehicle. After 48 h, the log10 cfu

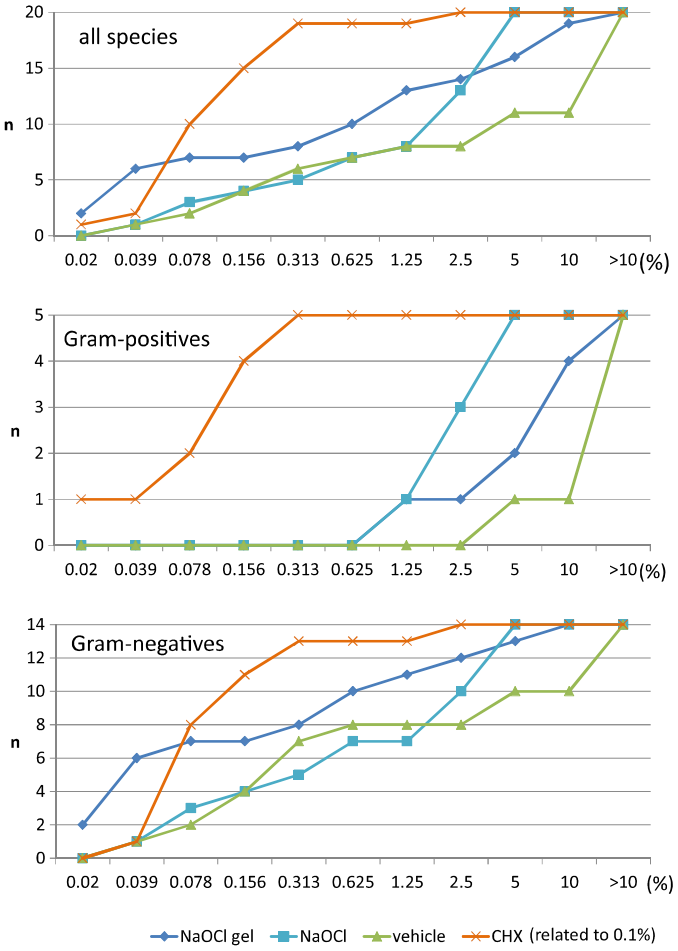

Fig. 1 MIC values of NaOCl gel including its components and chlorhexidine digluconate (related to the used formulations) against all species, Gram-positives and Gram-negatives

were the lower when the surface was coated with the activating vehicle (reduction about 2.7 log10 cfu in mean) than with NaOCl gel (reduction about 0.6 log10 cfu in mean) (Fig. 3).

Influence on 4-day-old biofilm

In these assays, 100 % of the formulations could act on the biofilms for 1 min before there was a dilution to 10 %.

The number of cultivable bacteria was reduced by about six log10 cfu after application of NaOCl gel, being one log10 cfu more than after CHX. When NaOCl only was applied, no cultivable bacteria were detected. The activating vehicle itself did not have any effect (Fig. 4a). These results did not correspond with those of the staining by crystal violet. Here, the highest values were measured for NaOCl ($p<0.01$ in comparison with control) followed by the untreated control and CHX. Lower staining was observed for the activating vehicle and the NaOCl gel ($p<0.05$, $p<0.01$ each in comparison with control) (Fig. 4b).

Staining with acridine orange showed a diffuse staining of the biofilm control, after application of CHX and the

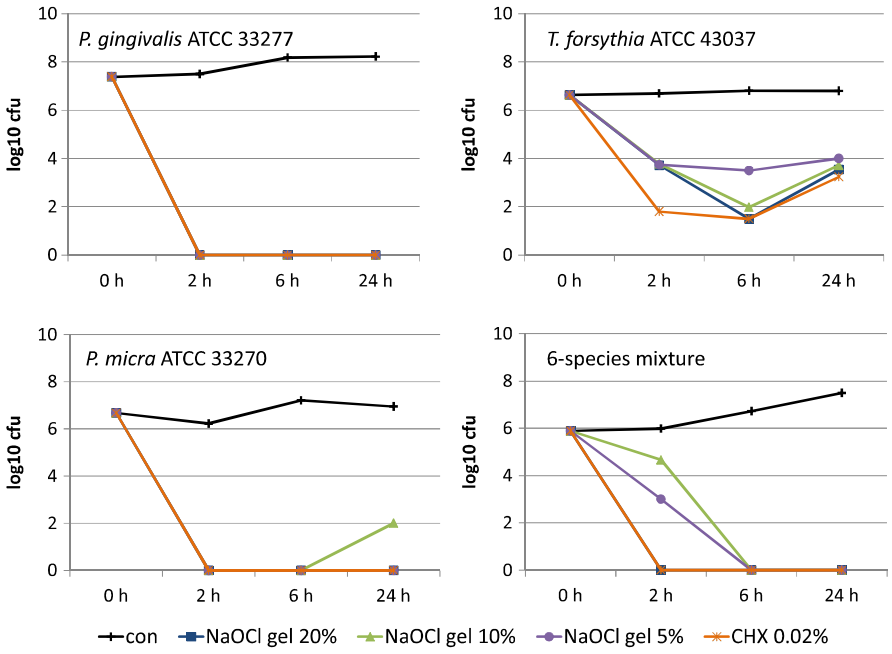

Fig. 2 Killing of *Porphyromonas gingivalis* ATCC 33277, *Tannerella forsythia* ATCC 43037, *Parvimonas micra* ATCC 33270, and a 6-species mixture by different concentrations of NaOCl gel and 0.02 % chlorhexidine digluconate (equivalent to 20 % of a 0.1 % formulation)

activating vehicle. The staining is less diffuse after NaOCl gel, whereas after NaOCl, only clearly defined structures (bacteria) are stained (Fig. 5).

In all SEM photographs, many bacteria were visible. In part, damaged bacterial surfaces were detected after application of NaOCl and NaOCl gel. The matrix seemed to be less after NaOCl in comparison with the activating vehicle (Fig. 6).

Discussion

In this in vitro-study, a sodium-hypochlorite gel was compared with a CHX solution which is still the gold standard in periodontal therapy. Growth inhibition and killing as well as the activity on a 6-species biofilm were evaluated.

MIC and MBC were determined by using standard procedures. The obtained values related to the available formulations revealed extremely low MICs of CHX and confirm findings from other studies [20, 21]. However, it should be mentioned that in the present study, CHX was tested without any additives. It is well known that additives may influence disadvantageously the antimicrobial activity of commercial CHX formulations [22, 23]. The activity of NaOCl gel differed between Gram-positive and Gram-negative bacteria, growth of Gram-negatives is inhibited by lower concentrations. Interestingly, in the present study, also the activating vehicle itself exerted certain antimicrobial activity. This selective

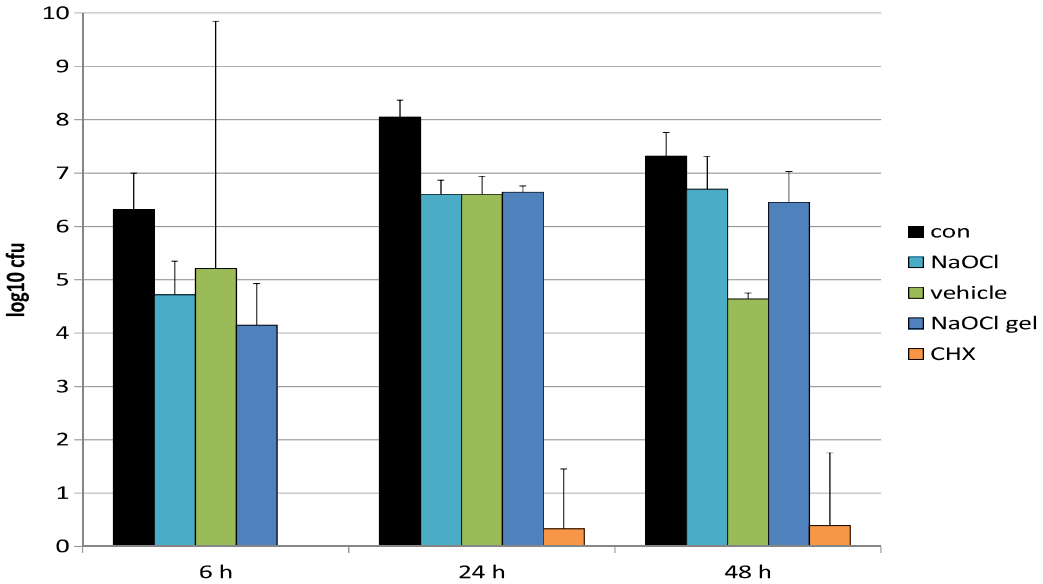

Fig. 3 Influence of NaOCl gel including its components and 0.1 % chlorhexidine digluconate on formation of a 6-species biofilm. Surfaces were coated with test substances for 1 h before bacterial suspensions were added

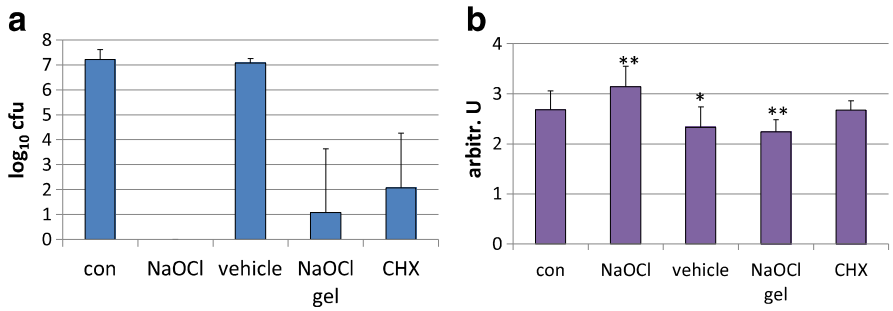

Fig. 4 Influence of NaOCl gel including its components and 0.1 % chlorhexidine digluconate on 4-day-old 6-species biofilm. NaOCl gel incl. its components and 0.1 % chlorhexidine digluconate were added for 1 h to the biofilm, thereafter they were diluted 1: 9 for 18 h, before cfu (**a**) were determined and matrix was stained by cristal violet (**b**)

inhibition may favor a more Gram-positive microbiota with *Actinomyces* spp., oral streptococci being in general more associated with periodontal health [24]. Moreover, it should be noted, that *Actinomyces* spp. are able to reduce nitrate to nitrite [25]. Killing of those bacteria by broad-spectrum antiseptics disturbs the physiological role of these oral bacteria in blood pressure control [26].

One important question to be answered was whether the application of NaOCl gel on a surface after mechanical plaque removal may prevent biofilm formation. This property is well known for chlorhexidine [27, 28], which is in part linked to its high substantivity [29]. Our in vitro study confirms the inhibition of biofilm formation for CHX, but for NaOCl gel no clear activity was seen. A slight inhibition (>1 log10 cfu) lasted only up to 24 h. However, NaOCl gel and in particular its component NaOCl clearly reduced vitality of a 4-day-old biofilm. The used mode simulated in vivo situation with an initial high (100 %) concentration of the compounds followed by a dilution.

The overwhelming activity of NaOCl on the 4-day-old biofilm in relation to the action against planktonic bacteria suggests an interference with the biofilm matrix. Staining of the biofilm mass confirmed reduced values after NaOCl gel. Crystal violet binds to negatively charged cell surface molecules and extracellular polysaccharides as a component of the biofilm matrix [30, 31]. After NaOCl, the values were higher about this unexpected finding can only be speculated. One explanation might be the exposure of hidden cell molecules to the surface or it might be a non-specific effect caused by the alkaline NaOCl.

Biofilm matrix consists of different components. Carbohydrates and proteins provide three-dimensional architectural integrity [32], enzymatic cleavage of matrix components is discussed to effect biofilm dispersal. Moreover, extracellular DNA as an important component of biofilm matrices [33] is essential in stress relaxation of biofilms [34]. Its degradation represents also an interesting approach for biofilm control [35]. NaOCl removes proteins; this property is well

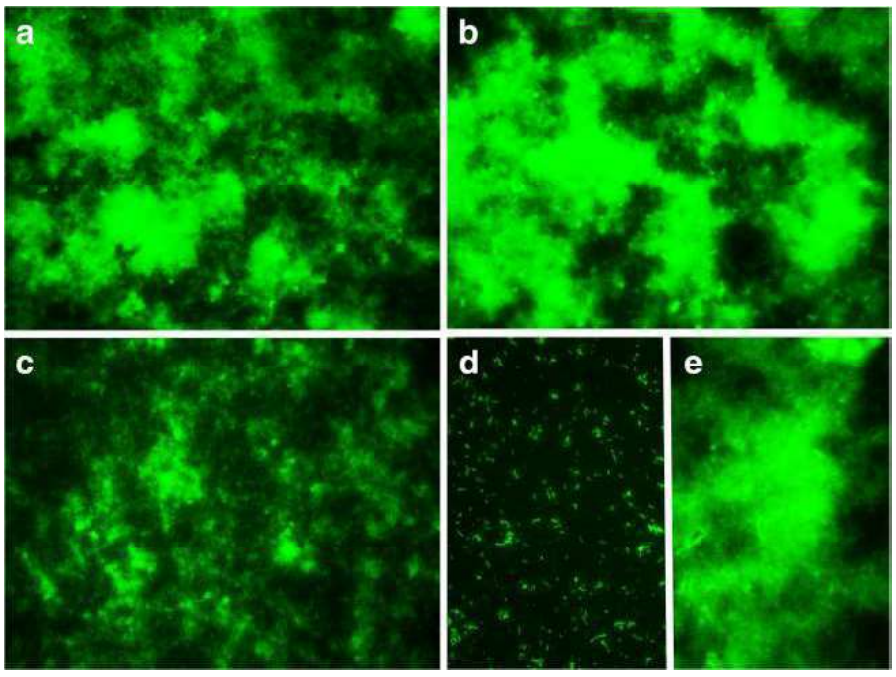

Fig. 5 Staining of DNA in a 4-day-old biofilm without (**a**) and after addition of 0.1 % chlorhexidine digluconate (**b**), NaOCl gel (**c**), and its components NaOCl (**d**) and activating vehicle (**e**) for 1 h and after dilution 1: 9 for 18 h

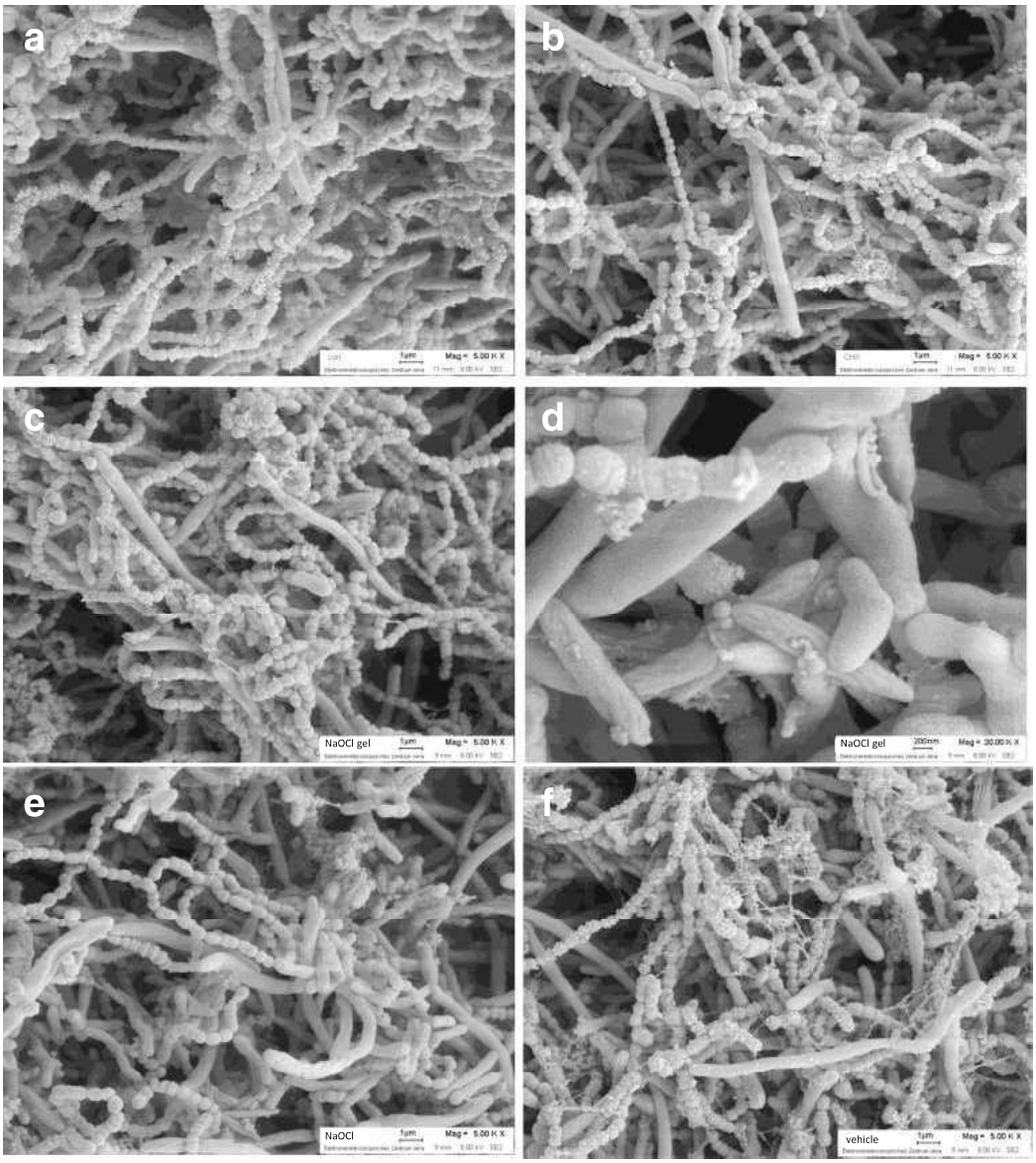

Fig. 6 Scanning electron microscopy photographs of a 4-day-old biofilm without (**a**) and after addition of 0.1 % chlorhexidine digluconate (**b**), NaOCl gel (**c**, **d**) and its components NaOCl (**e**), and activating vehicle (**f**) for 1 h and after dilution 1: 9 for 18 h

described when applied to dentin in caries [36] or endodontic lesions [37]. Besides of proteins, NaOCl may target extracellular DNA in biofilm matrix. It is very efficiently used in laboratories to decontaminate DNA [38]. In this study, staining with acridine orange was made. Acridine orange stains single-stranded and double-stranded DNA [39]. DNA staining of our biofilms may underline that extracellular DNA but not the intracellular bacterial DNA is destroyed by NaOCl [39]. Recently, by using eDNA extraction, it was shown that enzymatic treatment with DNase I was not able to affect biofilm matrix in a two-species biofilm model [40]. The results indicate NaOCl gel as an interesting approach in combating biofilm-associated diseases; further research should analyze its interference with biofilm matrix in more detail.

Both fluorescent staining as well as SEM photographs shows bacteria also in the NaOCl-treated biofilms. It can be suggested that bacteria are not viable anymore. In particular, a clear damage was also visible on coccoid species which might be of interest as NaOCl had high MIC values against both species included in the biofilm assays. On the other hand, the exposure of biofilms to antimicrobials may lead to formation of dormant persisters [41]. The presence of persisters cannot be excluded, since the time after exposure to high concentrations of antimicrobials might be too short to stimulate multiplication of persisters visible as cfu on agar plates.

Since several years, sodium hypochlorite gel has been proven to be effective in removal of dentin caries and despite the fact that treatment time was longer, the patients preferred this chemomechanical treatment to killing [42]. Furthermore, the data about its application in periodontal treatment are extremely rare, and long-term studies are still missing. A study including 12 participants did not find any benefit in terms of decrease of instrumentation time, number of strokes to provide a calculus-free surface, or subgingival calculus removal when sodium hypochlorite gel was applied adjunctive to SRP [43]. Similarly, the use of sodium hypochlorite gel as an

adjunct to SRP presented no advantage for smear layer removal over scaling alone [44].

In summary, the present in-vitro study has shown that the new NaOCl gel acts antimicrobial in particular against Gram-negative species associated with periodontitis. Despite the fact that the NaOCl gel has failed to eliminate a multi-species biofilm, the vitality was clearly reduced, and the matrix altered pointing to its high potential as an additive in mechanical therapy of periodontal disease.

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Conflict of interest The authors declare that they have no competing interests.

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