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ORIGINAL ARTICLE

Connective tissue graft plus resin-modified glass ionomer restoration for the treatment of gingival recession associated with non-carious cervical lesions: microbiological and immunological results

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Abstract
Objectives It was previously reported the clinical results of placing subgingival resin-modified glass ionomer restoration for treatment of gingival recession associated with non-carious cervical lesions. The aim of this study was to evaluate the influence of this treatment on the subgingival biofilm and gingival crevicular fluid (GCF) inflammatory markers.
Materials and methods Thirty-four patients presenting the combined defect were selected. The defects were treated with either connective tissue graft plus modified glass ionomer restoration (CTG+R) or with connective tissue graft only (CTG). Evaluation included bleeding on probing and probing depth, 5 different bacteria targets in the subgingival plaque assessed at baseline, 45, and 180 days post treatments, and 9 inflammatory mediators were also assessed in the GCF.
Results The levels of each target bacterium were similar during the entire period of evaluation ($p>0.05$), both within and between groups. The highest levels among the studied species were observed for the bacterium associated with periodontal health. Additionally, the levels of all cyto/chemokines analyzed were not statistically different between groups ($p>0.05$).
Conclusion Within the limits of the present study, it can be concluded that the presence of subgingival restoration may not interfere with the subgingival microflora and with GCF inflammatory markers analyzed.
Clinical relevance This approach usually leads to the placement of a subgingival restoration. There is a lack of information about the microbiological and immunological effects of this procedure. The results suggest that this combined approach may be considered as a treatment option for the lesion included in this study.

Keywords Gingival recession/surgery · Surgical flap · Cementoenamel junction · Glass ionomer cement · Tooth abrasion

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Introduction

Gingival recession, the apical shift of the marginal soft tissue and exposition of the root surface, is frequently associated

with cervical wear. Previous studies have reported that about 50% of gingival recessions are associated with non-carious cervical lesions [1, 2]. The association between non-carious cervical lesions and gingival recession, occurring concurrently in the same tooth, leads to a combined defect that may have a different prognosis regarding soft tissue coverage after periodontal surgery when compared to intact roots [3, 4].

Recently, a multidisciplinary approach to deal with this condition has been proposed in order to optimize the final esthetic outcome [3–6]. In these studies, gingival recessions associated with non-carious cervical lesions were treated with either a coronally advanced flap or connective tissue graft (CTG) plus a restoration of the cervical lesions using either resin-modified glass ionomer or composite resin. After the restoration was performed, a periodontal flap or connective tissue graft was placed on top of it, and after the healing period, 50% to 80% of the combined defect length was covered by soft tissue, meaning that at least half of the entire restoration length was located subgingivally. Although the apical margins of the restorations were located subgingivally after the treatment, these studies reported healthy tissue around the restorations.

Several studies reported the negative influence of restorations on periodontal tissues [7–9]. This negative influence may be related to the type of restorative material, presence of overhangs and surface roughness, and restorative margin location (subgingival placement). The subgingival restoration has been related to more gingival inflammation, bleeding on probing, and clinical attachment loss [8, 10], mainly due to the increase of subgingival biofilm formation and the alteration of its composition [11].

Recently, the influence of subgingival restorations made of composite resin and resin-modified glass ionomer on subgingival biofilm was evaluated [12]. In this study, the combined defect (gingival recession associated with non-carious cervical lesions) was treated with coronally advanced flap plus one of the two restorative materials. The results showed that the restorations, regardless of the type of material used, may not negatively affect the composition of the subgingival biofilm when compared to a non-restored group. However, more evaluations of the influence of this type of approach on subgingival biofilm are needed to confirm this observation. Additionally, an assessment of the inflammatory cytokines and chemokines after this approach has not been conducted. Thus, the aim of the present study was to evaluate the influence of the subgingival resin-modified glass ionomer restoration (placed during the treatment of gingival recession associated with non-carious cervical lesion treated with connective tissue graft) on five specific target bacteria and on the gingival crevicular fluid inflammatory markers.

Materials and methods

The present study was conducted using samples from 34 combined defects (gingival recession associated with non-carious cervical lesions) included in the previously published study [3]. The previous study included 40 combined defects that were randomly assigned to receive either connective tissue graft plus resin-modified glass ionomer restoration (CTG+R group, N=20) or connective tissue graft alone (CTG, N=20). In the present study, subgingival plaque and GCF samples were collected and analyzed for 17 patients out of the CTG+R group and 17 from the CTG group; the remaining 6 were excluded from the present study due to lack of subgingival biofilm and the GCF samples. Prior to the beginning of the study, the consent form and the protocol of the study were approved by the Institutional Review Board of the University of Campinas (CEP-UNICAMP 50/2008). Informed consent was signed by each subject after a thorough explanation of the nature, risks, and benefits of the clinical investigation and associated procedures.

Study population

Thirty-four participants, 20 males and 14 females, aged 19 to 55 years (mean age 32.80±19.1 years), were included. The subjects were selected from a group of patients referred for periodontal treatment to the Graduate Clinic of the Piracicaba Dental School at the University of Campinas to participate in this study. The patients were selected from March 2006 to February 2007, according to the following eligibility criteria:

1. Presence of one Class I Miller gingival recession associated with non-carious cervical lesions 1–2 mm deep in maxillary canines or premolars
2. Non-smokers
3. Systemically and periodontally healthy
4. No contraindication for periodontal surgery and no need for antibiotic prophylaxis prior to surgery
5. Had not taken medications known to interfere with periodontal tissue health and healing (including antibiotics in the previous 6 months)
6. Probing depth ≤3 mm without bleeding on probing on the area of surgery
7. Tooth vitality, absence of restoration on the cervical area, and absence of severe occlusal interferences in the area to be treated
8. No previous periodontal surgery in the area

Patients who were pregnant or breastfeeding or reported the use of any type of antibiotics or anti-inflammatory medication during the period of observation were excluded (no one was excluded for these reasons).

The patients were referred for periodontal treatment based on their complaints (dentin sensitivity and/or esthetic concerns). Since a non-carious cervical lesion may be the consequence of a multifactorial process, including tooth structure loss caused by nonbacterial acids (erosion), traumatic toothbrushing (abrasion), and occlusal loading (abfraction) [13, 14], all patients were included in a pre-treatment program in order to eliminate the possible etiologic factors related to non-carious cervical lesions and gingival recession. Oral hygiene instructions with a non-traumatic brushing technique and a soft toothbrush were given to all patients. Patients were also encouraged to avoid excessive consumption of acidic beverages and foods. When necessary, selective grinding was performed to remove occlusal interferences on the teeth included in the study. Scaling, root planing, and crown polishing were performed as necessary.

Clinical assessments

After 30 days of this initial therapy, the following parameters were recorded: (1) full-mouth visible plaque index [15] (FMPI) and the presence or absence of visible plaque accumulation at the site included in the study (PI); (2) full-mouth sulcus bleeding index [16] (FMBI) and presence or absence of bleeding on probing at the site included in the study (BOP); and (3) probing depth (PD), assessed as the distance from the gingival margin to the apical end of the gingival sulcus. The probing depth and bleeding on probing were assessed using a manual periodontal probe (UNC Hu-Friedy, USA) and were taken at baseline, 45 days, and 6 months after surgery.

Prior to the beginning of the study, the examiner (MPS) measured the probing depth and relative gingival recession of all patients two times within 24 h, with at least 1 h between the examinations. The examiner was judged to be reproducible after fulfilling the pre-determined success criteria. The Kappa index was calculated to probing depth, resulting in 91% of reproducibility.

Surgical procedures

All of the surgical procedures were carried out by one operator (EAS). The sites were randomly assigned by flipping a coin (FFS) to control group or test group immediately before surgery. The control group received connective tissue graft (CTG group) and the test group was submitted to connective tissue graft plus a resin-modified glass ionomer restoration (CTG+R group).

Surgical procedures details have been described in details in the previous publication [3]. Briefly, after local anesthesia (Alphacaine −2% Lidocaine with 1:100,000 Epinephrine, DFL, Rio de Janeiro, RJ, Brazil), an intrasulcular incision was made at the buccal aspect of the involved tooth. Two horizontal incisions were made at right angles to the adjacent interdental papillae, 1 mm apically to the level of the coronal border of the non-carious cervical lesion, without interfering with the gingival margin of the neighboring teeth. Two oblique vertical incisions were extended beyond the mucogingival junction and a trapezoidal mucoperiosteal flap was raised up to the mucogingival junction. After this point, a split-thickness flap was extended apically, releasing the tension and favoring coronal positioning of the flap. In the CTG group, the root and non-carious cervical lesion were planed with a finishing bur (KG Sorensen 9803FF—São Paulo, Brazil) and curettes until the tooth surface became smooth. In the CTG+R sites, a sterile rubber dam was placed to isolate the operative field, and the non-carious cervical lesion restoration was performed with resin-modified glass ionomer cement following the manufacturer's instructions (Vitremer—3M ESPE—St. Paul, MN, USA). The restoration was performed in order to reestablish the entire defect caused by the cervical wear. The entire length of the non-carious cervical lesion was restored, and the original contour of the tooth was reestablished. Afterwards, the epithelium on the adjacent papillae was stripped away and the connective tissue graft harvested from the palate using a scalpel with parallel blades (1.5 mm distant from each other) placed in such a way as to cover all of the non-carious lesion (CTG, control group) or the restoration (CTG+R, test group). Then the flap was coronally positioned and sutured (6.0 Polygalactin 910 Ethicon INC—São José dos Campos, Brazil) to completely cover the graft.

Postoperative care

Patients were instructed to take analgesics (500 mg sodium dipyrone every 6 h for 2 days) and to discontinue toothbrushing around the surgical sites during the initial 30 days after surgery. During this period, plaque control was achieved with a 0.12% chlorhexidine solution rinse used twice a day. After this period, gentle toothbrushing with a soft-bristle toothbrush was allowed.

Sutures were removed after 7 days, and the patients were enrolled in a periodontal maintenance program (professional plaque control and oral hygiene instruction) weekly during the first month and then monthly for 6 months.

Sample collection

Subgingival biofilm samples were collected from the buccal aspect of the sites that received CTG+R and from the sites that received CTG, to be used as a control group. The collection of the subgingival biofilm was performed at baseline (prior to the surgical procedure), 45 days, and 6 months

after the procedure. Following the careful removal of supragingival biofilm, areas were isolated with sterilized cotton rolls, and gently dried. A sterile paper point (No. 35) was inserted into the bottom of the gingival sulcus for 45 s. The paper points were placed into sterile tubes containing 300 ml of reduced transport fluid [17].

Gingival crevicular fluid (GCF) was collected from the same sites of microbiological sampling at the final follow up (6 months after the procedure). After subgingival biofilm collection, the GCF was collected by placing filter paper strips (Periopaper, Oraflow, Plainview, NY, USA) into the pocket until a slight resistance was perceived and then left in place for 45 s. Immediately, the volume of the sample was measured with the aid of a calibrated electronic gingival fluid measuring device (Periotron 8000, Oraflow, Plainview, NY, USA). After volume measurements, the strips were placed into sterile tubes containing 300 ml of phosphate-buffered saline with 0.05% Tween-20 [18]. Strips contaminated by visible blood were discarded. All samples (subgingival biofilm and GCF) were immediately stored at −20°C. Only one examiner (MPS) was responsible for the clinical measures as well as all samples collections.

Microbiological evaluation

The microbiological evaluation and the cytokine analyses were performed in the College of Dentistry at the University of Florida from November of 2008 to July of 2009. The TaqMan fluorogenic real-time PCR detection and quantification were based on the amplification of variable regions of the 16S rRNA genes of *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Streptococcus sanguinis*. The primers and the probes sequences of the bacteria are listed in Table 1 [19]. *S. sanguinis* sequence was obtained from Gene Bank and probe and primers were designed using a software.

Paper points were first sonicated for 15 s. Bacterial DNA was extracted from subgingival biofilm using QIAamp Mini Kit following the manufacturer's instructions (Qiagen, Germany). Briefly, a 200-μL aliquot from each of the subgingival plaque samples was used. 20 μL of protease was pipetted into a fresh 2.5 mL tube. In the same tube, 200 μL of the subgingival plaque sample and 200 μL of AL buffer were added and then incubated at 56°C for 10 min. After the incubation, 200 μL of 100% ethanol was added and the solution was mixed. Afterwards, the solution was placed into a spin column provided in the kit and centrifuged at 8,000 rpm for 1 min. The filtrate was discarded and the column spin was washed twice with 500 μL of AW1 buffer and once with 500 μL of AW2 buffer. The DNA was eluted in 50 μL of AE buffer.

Table 1 qPCR primers and probes for detecting bacteria

Bacteria		Amplicon size in bp (reference)
Porphyromonas gingivalis		
Forward	5′-TGGGACTTGCTGCTCT TGCTATG-3′	194 [19]
Reverse	5′-GATGGCTTCCTGCTGT TCTCCA-3′	
Probe	5′-/56-FAM/CAAAGACAA CGAGGCAGA	
Tannerella forsythia		
Forward	5′-GCGTATGTAACCTGC CCGCA-3′	149 [19]
Reverse	5′-CCGTTACCTCACCAA CTACCTAATG-3′	
Probe	5′-156-FAM/AGGGATAAC CCGGCGAAAGTCGGA	
Prevotella intermedia		
Forward	5′-AGACGGC CTAATACCCGATGTTG-3′	105 [19]
Reverse	5′-TTACCCGCACCAACA AGCTAATCAG-3′	
Probe	5′/56-JOE N/ TGGCATCTGACGTGGAC	
Fusobacterium nucleatum		
Forward	5′-GCGGAACTACAAGTGTA GAGGTG-3′	175 [19]
Reverse	5′-GTTCGACCCCCAACACC TAGTA-3′	
Probe	5′/56-JOE N/AATGCCGATG GGGAAGC	
Streptococcus sanguinis		
Forward	5′-GGACAGAGGTGACAGG TGGT-3′	124 Gen Bank ATCC 10556
Reverse	5′-GTCTCGCTAGAGTGCC CAAC-3′	
Probe	5′/56-JOE NCAGCTCGTGT CGTGAGATGT	

Real-time TaqMan® assay

The TaqMan® fluorogenic real-time PCR detection system was used to determine number of DNA copies for each species [20]. Real-time PCR was performed using the iQ5 real-time PCR detection system (Bio-Rad Laboratories Inc, Hercules, CA). The PCR reaction was performed as individual assays for each species studied. For each species tested, the TaqMan PCR assay was performed with a final volume of 25 μL of reaction mixture, containing 2 μL of extracted clinical sample, 12.5 μL of iQ Supermix PCR Master Mix (Bio-Rad Laboratories Inc, Hercules, CA) 1 μL of each primer (forward and reverse) and TaqMan probe, and 7.5 μL of RNAse/DNAse free water. The PCR cycling program included 3 min at 95°C and then 40 cycles,

with each cycle consisting of three steps at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Absolute quantification of the TaqMan PCR assay was determined by serial dilution of the five bacteria target DNA in the range of 10^9–10^2 copies per μL. The standard curves were used to convert cycle threshold scores into the number of bacterial cells using controls with known amounts of bacterial-specific DNA.

Immunological evaluation

Cyto/chemokine levels were determined using a multiplexed bead immunoassay. Aliquots of each GCF sample were assayed using a commercial human 10-plex kit (Millipore Corporation, Billerica, MA, USA) in order to evaluate the levels of nine inflammatory markers: IL1β, IL4, IL5, IL6, and IL10, Interferon gamma-induced protein 10 kDa (IP10), macrophage inflammatory protein 1α (MIP1α), monocyte chemotactic protein 1α (MCP-1α), and tumor necrosis factor α (TNFα). The assay was performed according to the manufacturer's instructions. Briefly, the 10-plex multi-cytokine standard was resuspended in 250 μL deionized water, and serial dilutions were performed in order to achieve quality control. Afterwards, the wells of 1.2-μm filter membrane 96-well microtiter plates were pre-wetted with 200 μL of assay buffer and removed after 10 min. Assay buffer (25 μL) was added into the wells again, and 25 μL of standards and samples were added into the appropriate wells. Then 25 μL of the premixed microbeads was added to the wells and incubated for 2 h on an orbital plate shaker at room temperature and protected from direct light. The plates were washed twice with 200 μL assay wash buffer. Then 25 μL of detection biotin detector antibody was added per well. The plate was incubated for 1 h at room temperature on the plate shaker. Without washing, 25 μL/well of streptavidin–phycoerythrin solution was added and the plates were incubated for a further 30 min at room temperature on a plate shaker, protected from direct light. Then the plate was washed twice with 200 μL assay wash buffer, 125 μL of sheath fluid was added to the wells, and the plate was shaken for 5 min and analyzed using the Luminex 100 system (MiraiBio, Alameda, CA, USA). The concentrations of the samples (antigens in gingival crevicular fluid samples) were estimated from the standard curve using the standard proteins in the kit and expressed as pg/mL after adjusting for the dilution factor.

Statistical analysis

Descriptive statistics were expressed as the mean±standard deviation (SD), and counts of the bacteria were normalized by conversion to logarithmic values. The Shapiro–Wilk test was used to evaluate normality. The PD, FMPI, FMBI, and bacterial counts were examined by repeated measures of analysis of variance (Friedman test) followed by post hoc non-parametric test for multiple comparisons to evaluate the differences within groups among all periods. Mann–Whitney test was used to evaluate the differences between groups at each time interval. The frequency detection of each target bacteria was compared between groups using Chi-square analysis. The levels of the inflammatory markers showed normal distribution, and the *t* test was used to assess the difference between the test and control groups for each marker. Additionally, a post hoc power analysis was performed using the data of each bacterium concentration from test and control group. All analysis was carried out using SAS Software 2001—Release 8.2 (SAS Institute Inc., Cary, NC, USA). The experimental level of significance (α) was set at 0.05.

Results

Clinical results

All clinical parameters were previously reported [3]. Briefly, after the healing period, the mean restoration coverage was 70.0±13.85%. This means that about 70% of the entire restoration length was subgingivally located. Full-mouth bleeding on probing (FMBI) remained low during the entire study period. It was 14% at baseline and 16% at the 6-month for CTG group (control group) and 19% at baseline and 18.5% at the 6-month evaluation for CTG+R (test group). No bleeding on probing was observed either at the test sites or at the control sites included in the study in any evaluated period. Additionally, low levels of the FMPI were observed during the entire study period, maintained below 20%, indicating a good standard of supragingival plaque control during the study period. The probing depth presented a statistically significant increase for both groups. It changed from 1.2±0.3 to 2.1±0.71 mm in the test group ($p<0.05$) and 1.13±0.4 to 2.0±0.5 in the control group ($p<0.05$). The differences between groups were not statistically significant ($p>0.05$). A flow diagram of participants in the study is enclosed (Fig. 1). Table 2 shows the patients' characteristics at baseline. Table 3 shows the clinical parameters, and Table 4 shows the parameters regarding the lesion coverage for the two groups.

Microbiological results

The quantitative real-time PCR analysis results showed similar detection frequencies and total amount of each target bacterium between groups. When observed separately, all target bacteria were detected and, in general, the frequency of detection and their mean concentration remained constant during the evaluated periods. No statistically significant differences were observed in the intragroup analysis or in

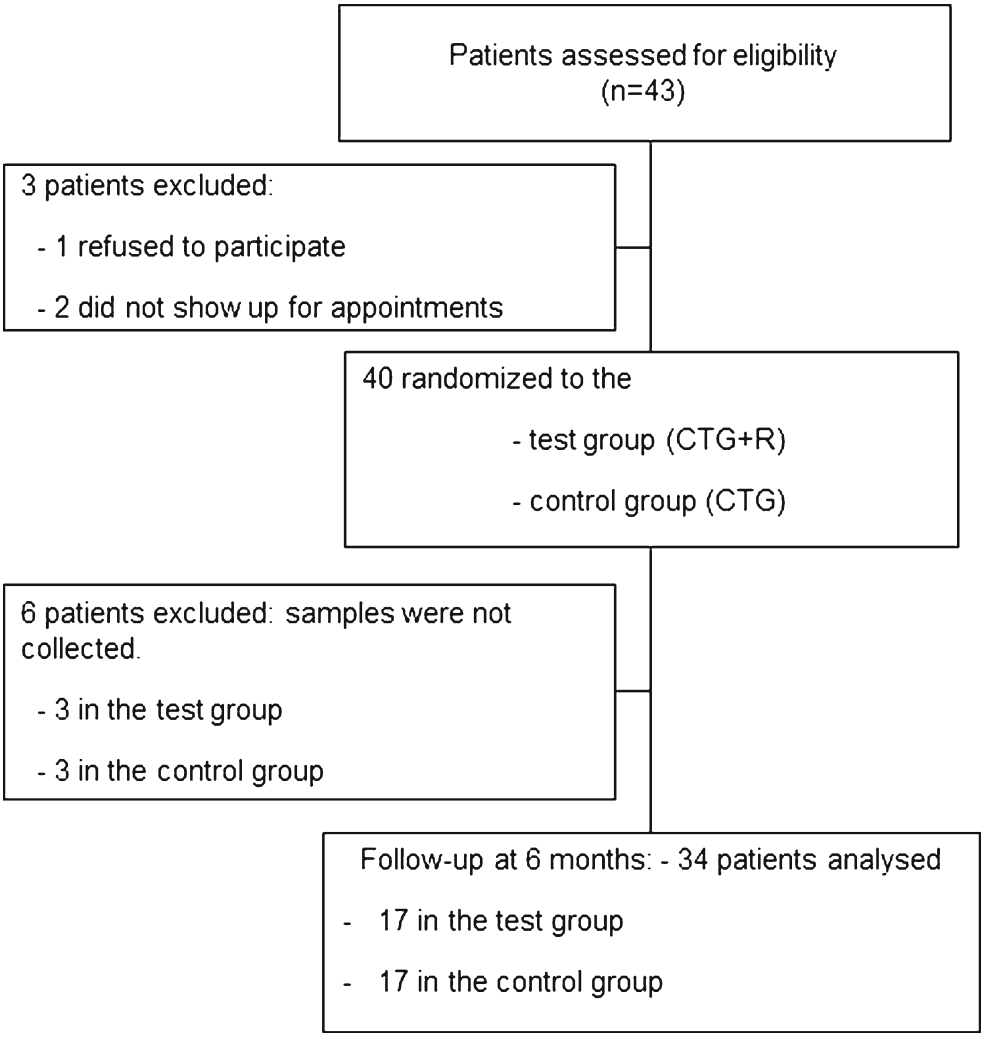

Fig. 1 Flowchart for the study patients. *CTG* connective tissue graft group, *CTG+R* connective tissue graft plus restoration group

the intergroup analysis. *S. sanguinis* was the most frequent species detected, reaching 100% of detection in both groups in all periods, while *P. gingivalis* and *P. intermedia* were the less-frequently detected bacteria. A reduction trend was observed in the detection frequency of some bacteria over time. *P. gingivalis* was detected in 47.05% of the sites in the test group and 41.17% in the control group at the baseline ($p>0.05$). After 6 months, this bacterium was detected in 41.17% and 35.3%, respectively ($p>0.05$). *P. intermedia* was detected in 35.29% of the sites in the test group and in 29.41% of the control group ($p>0.05$). After 6 months, this bacterium was detected in 29.41% and 23.52%, respectively ($p>0.05$). *T. forsythia also* showed a trend to reduce its frequency of detection as well. It was detected in 64.7% of the sites in the test group and in 52.94% in the control group at the baseline. After 6 months, *T. forsythia* was found in 52.94% of the test group and in 41.17% of the control group. However, these intragroup differences were not statistically significant ($p>0.05$). Figure 2 shows the frequency of detection of each target bacteria.

In terms of absolute quantification of target bacteria, *S. sanguinis* presented the highest amount when compared to the other targets, while the lowest were *T. forsythia* and *P. intermedia*. When the test group was compared to the control group, no statistically significant difference was observed at any time point between groups ($p>0.05$). When intragroup

Table 2 Patients characteristics at the baseline ($N=34$)

	Control group	Test group
Age range (mean ± SD)	22–51 (34.4±12.2 years)	19–55 (39.4 20.4 years)
Gender	9 males and 8 females	11 males and 6 females
FMPI	19.4%	18.5%
FMBI	16%	18,5%
Teeth involved	Canines: 10 (58.8%)	Canines: 9 (52.9%)
	1PM: 5 (29.4%)	1PM: 6 (35.3%)
	2PM: 2 (11.8%)	2PM: 2 (11.8%)

FMPI
full-mouth visible plaque index at baseline, *FMBI* full-mouth sulcus bleeding index at baseline

Table 3 Clinical parameters of control and test sites in the different time periods (N=34)

Period	Baseline		45 days		180 days	
	Control	Test	Control	Test	Control	Test
% of teeth presenting BOP	0	0	0	0	0	0
% of teeth presenting visible plaque	0	0	0	0	0	0
PD±SD (mm)	1.13±0.4	1.2±0.3	1.77±0.7[a]	1.82±0.5[a]	2.0±0.5[a]	2.1±0.71[a]

Intergroup statistically significant difference (Mann–Whitney)

BOP bleeding on probing, *PD* probing depth, *SD* standard deviation

[a] Intragroup statistically significant difference (Friedman test)

analysis was performed, both test and control groups maintained similar amounts of each target during the entire study period, with no statistically significant differences (p>0.05). Figure 3 shows the amount of each bacterium for both groups. The power analysis showed that, with the number of subjects in each group in the present investigation, the minimum power value found was 80% (*S. sanguinis*=84%, *P. gingivalis*=80%, *P. intermedia*=88%, *T. forsythia*=83%, *F. nucleatum*=91%).

Immunological results

The GCF from the test and control group sites was collected only at the final evaluation (6 months). Intergroup analyses demonstrated that there was no statistically significant differences in the GCF volume collected between groups (mean volume of 0.33±0.16 μL for the test group and 0.40±0.21 μL for the control group; p>0.05). Additionally, there were no statistically significant differences between groups when the concentration of each cyto/chemokine was analyzed (p>0.05). Figure 4 shows the quantities of the target cytokines for test and control sites at 6 months

Discussion

Recently, gingival recession associated with non-carious cervical lesions has been treated using a multidisciplinary approach (periodontal and restorative) [3–6]. The final result of this type of approach leads to subgingival placement of the restorative material. As shown in the literature, this condition has the potential to induce gingival inflammation and pocket formation by facilitating plaque accumulation through the tooth-restoration interface [8]. Conversely, the clinical results of those trials aiming to deal with gingival recession associated with non-carious cervical lesions using periodontal–restorative approach showed that, even if the apical margin of the restorations is located subgingivally after the healing period, no signs of inflammation, such as redness, suppuration, and bleeding on probing, were observed.

Table 4 Mean values and standard deviation for test and control groups

	Test group (n=17)	Control group (n=17)	P value
CLH	3.22±0.52 mm	3.27±0.68 mm	0.81
CLH coverage	77.59±20.15%	70.0±13.85%	0.2

P values were calculated by Mann–Whitney test to evaluate differences between groups

CLH non-carious cervical lesion height

These observations differ from those presented by other reports evaluating the impact of a variety of restorative materials and restoration procedures upon the periodontium. Some of these studies have claimed that subgingival dental materials may favor the initiation of gingivitis and periodontitis by local plaque accumulation [9, 21]. The discrepancy among the older reports and the present study may be due to the heterogeneity of the materials and restorations evaluated. Some of the older reports included crowns or other types of indirect reconstruction that have a tooth-restoration interface presenting a gap, which is usually greater than the gap found between tooth and adhesive fillings. This difference in the size of the tooth-restoration gap can allow plaque accumulation, which can favor the initiation of gingivitis. In addition, the flap elevation performed in the present study before the restorative procedure allowed proper isolation of the entire non-carious cervical lesion length and, as a consequence, a well-finished, well-polished filling without over-hanging could be achieved. Other possible reasons that can explain these differences include: biocompatibility of the material, frequent patient follow up with monthly prophylaxis, plaque control, and oral hygiene instructions. All these factors may have contributed to the healthy condition of the gingival tissue observed during the present study.

Despite the good clinical results of this multidisciplinary approach (periodontal and restorative), more evidence is needed to assess the safety of this procedure. Thus, the

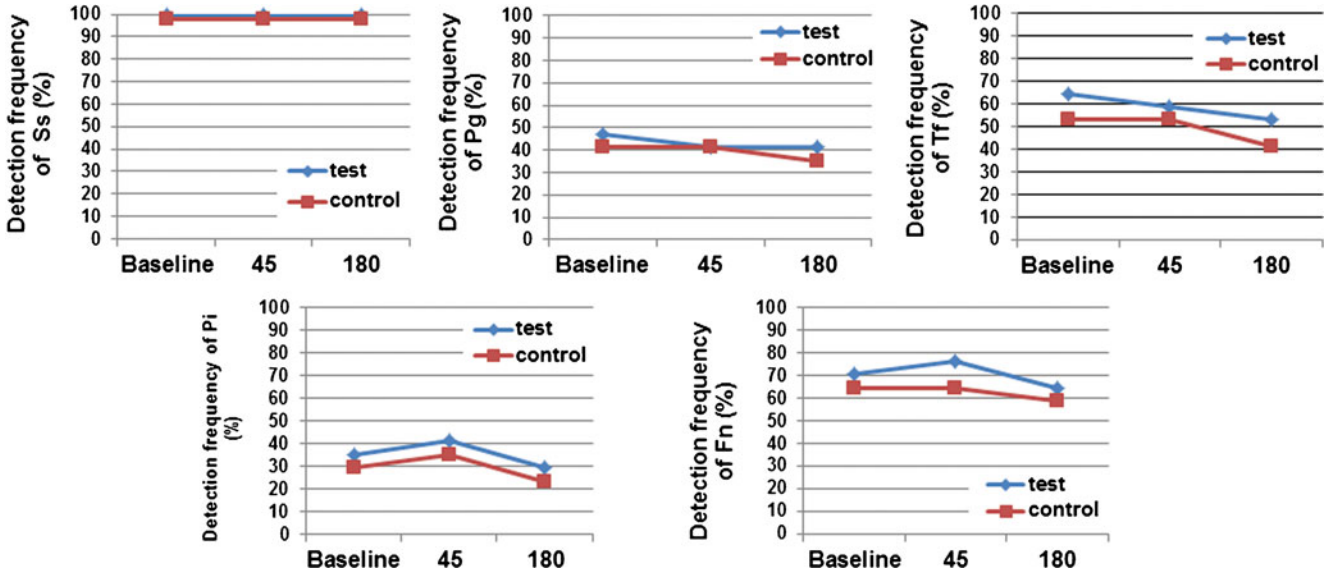

Fig. 2 Detection frequency of all target species in the test and control sites

aim of the present study was to evaluate the possible influence of this procedure on the subgingival biofilm and on the levels of inflammatory cytokines. The present study showed that the changes from the baseline in the DNA amount of each target bacteria were not statistically significant when the combined procedure was evaluated ($p>0.05$). When compared to the control group, the differences were also not statistically significant ($p>0.05$). These results may suggest that the presence of the subgingival modified glass ionomer restoration, coupled with an effective regimen of plaque control, may not have a negative impact on the subgingival biofilm accumulation. These results are also in agreement with those of a previous study [12].

When each target bacterium was observed separately, their concentrations remained similar during the entire period of observation. The target bacterium detected most frequently was *S. sanguinis*, which was observed in 100% of the test and control sites in all evaluated periods. The putative pathogenic periodontal bacteria were also detected, and their frequency of detection and concentration were also similar between groups and within each group during the observation period. These results are in accordance with those of a previous study that

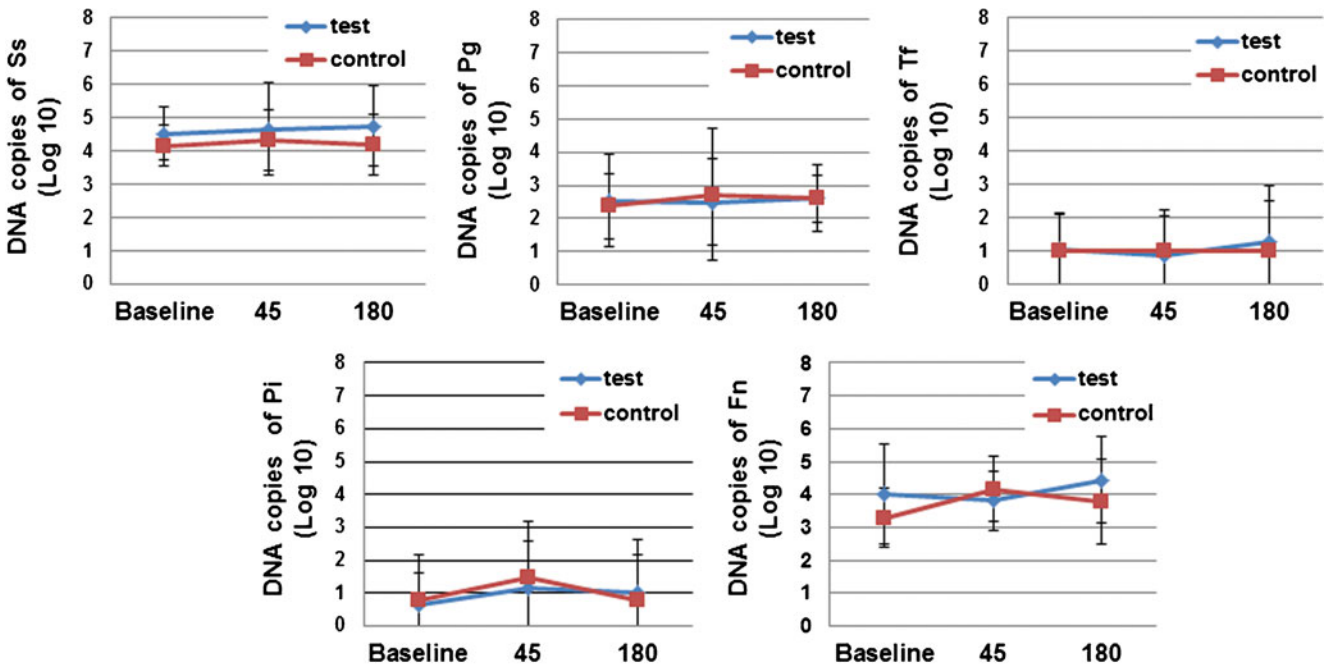

Fig. 3 DNA copy amounts for each target species in the test and control sites. No differences observed between or within groups ($p>0.05$)

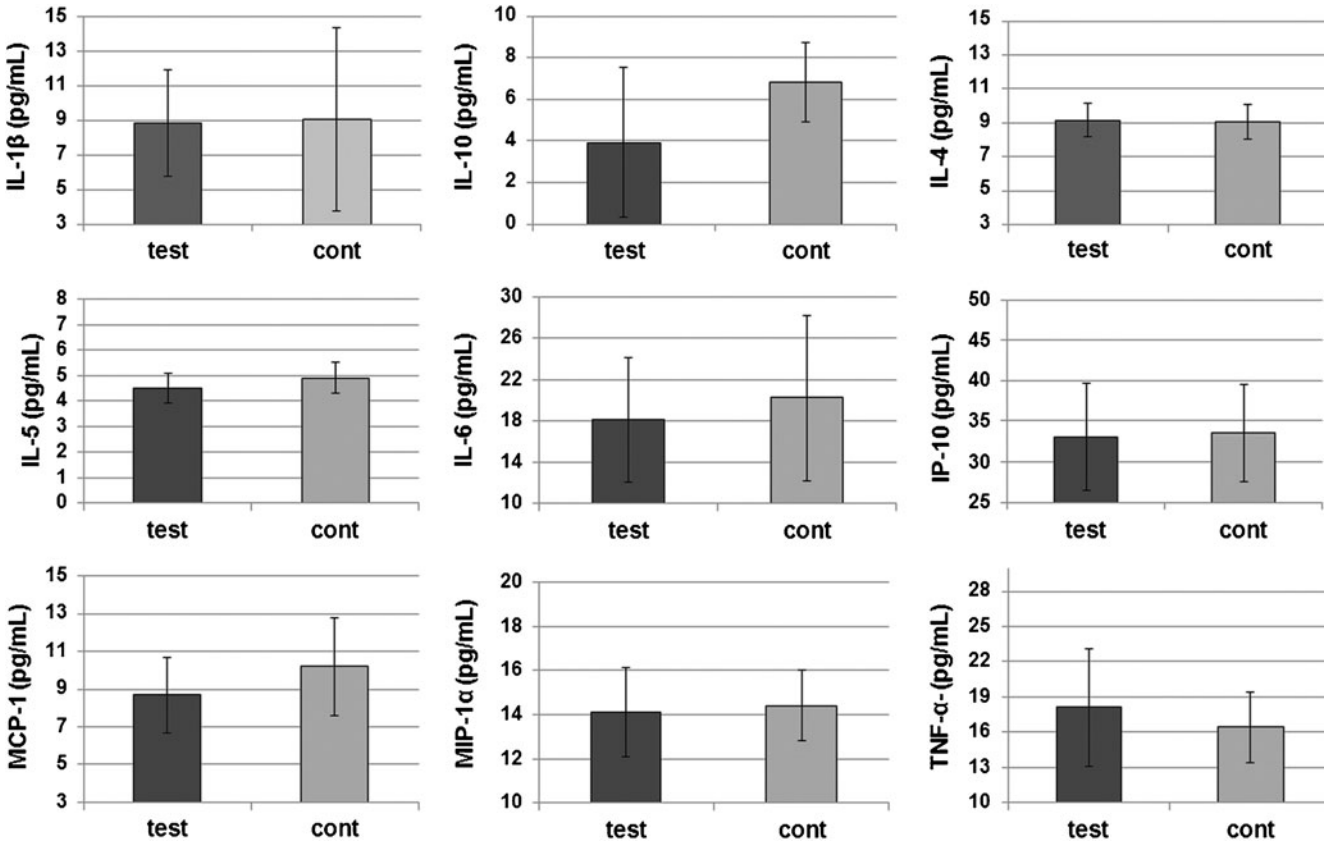

Fig. 4 Levels of each analyzed cytokines/chemokines in the test and control sites at 6 months post treatment. The differences observed were not statistically significant between groups ($p>0.05$)

evaluated the influence of composite resin and modified glass ionomer on subgingival biofilm using a DNA–DNA checkerboard [12]. These authors observed that the presence of the restoration margins into the subgingival area, regardless of the type of the material used, did not change the composition of the biofilm after 6 months and, as a consequence, healthy clinical parameters could be observed.

There are differences in the patterns of subgingival microbiota among populations and heterogeneity among periodontally healthy and diseased subjects. The extent to which these differences occur is not clear, and many factors, such as age, smoking, gender, gingivitis, and country of origin may play a role in the shifting of the microbiological profile. Thus, no single microbial profile can be associated with no or low levels of periodontitis. Rather, these subjects exhibit a wide range of quite distinct subgingival microbiota [22]. However, it is clear that some changes in the composition of the biofilm may indicate a high risk for inflammation development, such as higher counts of red complex bacteria. In the present study, the fact that the detection frequency and concentration of these bacteria remained similar during the 6-month study period may indicate that the restoration used to treat this specific condition may not alter the subgingival biofilm longitudinally for at least 6 months. Additionally, the higher concentrations and detection frequency of *S. sanguinis* observed corroborate with the previous statement since this bacterium is one of the major components of periodontally healthy subgingival plaque biofilm. This observation is in accordance with those of previous reports [22, 23]. Finally, the lack of differences in the microbial profile within each group also correlates with the healthy clinical measurements observed for these sites in all time-points, which corroborate with the hypothesis that the subgingival placement of the restoration may not affect integrity of periodontal tissues for at least 6 months post treatment, provided there is good plaque control by the patients.

Despite the absence of statistically significant difference in the microbiological parameters, one may argue that the periodontopathogenic bacteria colonization levels might be in higher levels than it should be expected for periodontally healthy subjects. This could be explained by the difference among populations around the world. In the Brazilian population, even in healthy subjects, about 35% of *P. gingivalis* prevalence has been observed, which is similar to our findings [24]. Additionally, other studies have provided information showing higher levels of periodontopathogenic bacteria in Brazilian population when compared to others

[25, 26]. The interpretation of the microrganisms counting should be made with caution. Rather than relying on the absolute concentration or prevalence of each bacterium, the association of subgingival bacteria (putative periodontopathogenic) with other beneficial microrganisms should be done. In addition, the longitudinal shifts of this microbial profile are important and should be monitored, along with long term changes in clinical parameters.

Several previous reports have shown that the levels of cytokines measured in the GCF increased in sites presenting periodontal disease when compared to healthy sites [27, 28]. The findings of the present study showed that both test and control groups showed similar levels of pro-inflammatory or anti-inflammatory markers. The anti-inflammatory cytokines, such as IL-4 and IL-10, have been reported as having a protective role, downregulating macrophage function and inhibiting the secretion of some pro-inflammatory cytokines [29]. It has been reported that IL-4 is found in higher concentrations in periodontally healthy sites when compared to diseased sites [30]. In the present study, both the test and control groups presented similar levels of these markers (p=0.48 for IL-10 and p=0.17 for IL-4). Other cytokines found in high concentrations in diseased sites, such as IL-1β, TNF-α, MIP-1α, and MCP-1, presented similar amounts in the two groups in the present study. These results suggest that the presence of subgingival modified glass ionomer restoration may not interfere with the levels of inflammatory markers in GCF. The lack of differences in these levels between the two groups coupled with healthy clinical and microbiological parameters observed here may suggest subgingival placement of glass ionomer with these surgical procedures is safe for the periodontium.

However, caution needs to be exercised when interpreting these results. Although our findings correlate with the absence of clinical inflammation presented here and in other previous reports, the cytokine levels were evaluated only at 6 months post treatment, thus changes in these parameters could not be assessed over time. Additionally, the small sample size present in this study should be addressed. Despite the relatively small sample size in the present investigation, the number of subjects per group was enough to reach an 80% power value to detect a clinically significant change in subgingival bacterial composition, detected by a post hoc power analysis performed. Finally, it also should be stressed that the data in the present study only reflect the results for this specific condition (i.e., gingival recession associated with non-carious cervical lesions). Other conditions, such as treatment of root caries and root fractures may yield different results from the ones obtained in the present study.

Within the limits of the present study, it can be concluded that the presence of subgingival modified glass ionomer restoration as a result of the performed periodontal/restorative approach to treat gingival recession associated with non-carious cervical lesions may not negatively interfere with the subgingival microflora and with inflammatory markers present in the GCF after an observation period of 6 months. Taken together, the microbiological and inflammatory levels observed in the present study corroborate with the clinical findings previously reported and may provide extra support for this surgical approach. Other trials, with larger samples, to evaluate different materials and changes in inflammatory markers over time are recommended.

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