Clin Oral Invest (2007) 11:61–68
DOI 10.1007/s00784-006-0087-7

ORIGINAL ARTICLE

Comparison of the proliferative activity in gingival epithelium after surgical treatments of intrabony defects with bioactive glass and bioabsorbable membrane

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Received: 19 July 2006 / Accepted: 18 October 2006 / Published online: 17 November 2006

Abstract Guided tissue regeneration is based on preventing the more rapidly proliferating epithelium from growing into the periodontal defect after surgical procedures incorporating barrier membranes. The aim of this study was to compare the proliferative activity of gingival epithelium using proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation after surgical treatments with bioactive glass graft material and bioabsorbable membrane. Using split mouth design, 20 intrabony defects were randomly assigned treatments with bioactive glass (BG group) or bioabsorbable membrane (BM group). Gingival biopsies were taken at preoperative and postoperative 12 weeks. After histological processing, the number of the inflammatory cells was measured in hematoxylin and eosin-stained sections; PCNA expression was determined in immunohistochemically-stained sections. At postoperative 12 weeks, the number of the inflammatory cells was significantly decreased ($p<0.01$), PCNA expression was significantly increased ($p<0.001$) in both treatment groups compared to baseline data. There was no significant difference in PCNA expression between baseline values of two groups ($p>0.05$), while at postoperative 12 weeks, increase in BG group was significantly greater than that in BM group ($p<0.001$). These results suggest that epithelial cell proliferation is more prominent after treatment of intrabony defects with bioactive glass compared to the treatment with bioabsorbable membrane.

Keywords Guided tissue regeneration · Bioactive glass · Bioabsorbable membrane · Long junctional epithelium · Proliferating cell nuclear antigen

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Introduction

Periodontal disease is a slowly progressing infectious disease resulting in inflammatory destruction of the alveolar bone as well as the soft tissue attachment loss of the teeth [44]. Periodontal regeneration is defined as the reconstruction of the damaged periodontium as evidenced histologically in the formation of new cementum, periodontal ligament, and alveolar bone to a previously diseased root surface [4, 11, 18, 35]. The interaction between the hard and soft tissues makes periodontal wound healing a complex process [19]. The epithelial cells demonstrate the highest capacity of proliferation among periodontal tissues involved in wound healing [7]. The proliferation and migration of basal epithelial cells of the oral and sulcular epithelium cause the formation of a long, nonkeratinized junctional epithelium which prevents the migration of the cells from the periodontal ligament and alveolar bone [13].

Several treatment procedures incorporating several types of bone graft materials, barrier membranes, combined

approaches, and growth factors have been used to treat intrabony defects with the goal of attaining periodontal regeneration [2, 20, 26, 28, 30, 37, 38]. Recently, an oily calcium hydroxide suspension has also been supposed to support regeneration of the periodontal tissues [34, 35, 39]. Guided tissue regeneration (GTR), achieved using barrier membranes, is one of the most widely used treatments that increase regeneration of periodontal tissues [16, 18]. The main function of the membrane is to provide a selective isolated space which accelerates repopulation of cells from the periodontal ligament and bone while preventing the more rapidly proliferating epithelium from growing into the periodontal defect [12, 28]. In recent years, alloplastic synthetic bone graft materials have also been used increasingly in the treatment of intrabony defects [30]. According to the 1996 World Workshop in Periodontics, "On a histologic basis, alloplasts act almost exclusively as biologic fillers inducing little bone fill and very limited if any periodontal regeneration" [11]. Typically, the healing after treatments with these graft materials evidences a long junctional epithelium and minimal bone formation [3]. However, bioactive glass, alloplastic synthetic bone grafting material, has been reported to have an ability to enhance clinical attachment in periodontal lesions [28]. It has been demonstrated in nonhuman primates that the use of bioactive glass induces a significant increase in newly formed cementum and attachment while preventing apically directed growth of the junctional epithelium [10, 17, 43]. However, human histological evidence has shown healing with a long junctional epithelium with no evidence of new cementum or new attachment [31].

Comparing the type of healing from a viewpoint of the proliferative activity in gingival epithelium may be important as healing by a true regeneration via new attachment or healing by repair via a long junctional epithelium may demonstrate similar clinical and radiological results [21]. To our knowledge, there are currently no studies comparing the proliferative activity of epithelium after the surgical treatments of intrabony defects with bioactive glass and barrier membranes.

In humans, proliferative activity can be determined by counting the cells in the mitotic phase [8]. Subsequently, certain nuclear antigens have been identified in proliferating cells [23, 25, 29, 41]. Proliferating cell nuclear antigen (PCNA) is one of the nuclear antigens which is defined as a 36-kDa auxillary protein to DNA polymerase delta [6, 15]. Monoclonal antibodies to these nuclear antigens are found to be effective in obtaining information about the proliferative characteristics of various types of tissues [14]. This study was designed to compare for the first time the proliferative activity of gingival epithelium using PCNA as a marker of cell proliferation after surgical treatments of intrabony defects with bioactive glass and bioabsorbable membrane. The general hypothesis to be tested was that the proliferative activity of the epithelium can be an indicator to evaluate the type of healing after regenerative periodontal treatments.

Materials and methods

Study population

Ten systemically healthy patients (six males and four females) with a mean age 42.70±2.16 years (range 33 to 55 years) exhibiting radiographic evidence of bone loss were recruited for the study. The criteria needed for inclusion consisted of patients having similar paired vertical interproximal osseous defects. The exclusion criteria were systemic diseases (e.g., diabetes mellitus, cancer, HIV, metabolic diseases), chronic high dose steroid therapy, radiation or immunosupressive therapy, pregnancy, lactation, smoking, orthodontic treatment, extensive carious lesions, and medication in the 6 months preceding the study or during the study.

Initial periodontal therapy which consisted of oral hygiene instruction, full-mouth scaling and root planing, and occlusal adjustment when indicated was performed on all the patients. Four to 6 weeks after completion of initial periodontal therapy, a periodontal reevaluation was performed. Using a split mouth design, 20 paired interproximal intrabony defects with pocket depth 6 mm or over, were randomly treated with either bioactive glass (BG group) or bioabsorbable membrane (BM group). Randominization was done in each case during the surgical treatment before the allocation of the materials by a coin toss.

After receiving information on the study, the patients signed a consent form indicating their agreement to participate in the study. The study protocol and consent form were approved by the University Institutional Review Board.

Clinical measurements and surgical procedure

Probing pocket depth (PPD), clinical attachment level (CAL), plaque index (PI) [36], and gingival index (GI) [22] were assessed immediately before surgery and at 12 weeks after surgery by force-controlled Florida Probe (Florida Probe, FL, USA). The measurements were repeated in six areas per tooth: mesiobuccal, distobuccal, midbuccal, mesiolingual, distolingual, and midlingual. Clinical measurements were done by the same calibrated examiner who was blinded to the treatment modality.

All the periodontal surgical procedures were performed on an outpatient basis under aseptic conditions by two experienced periodontal clinicians under local anesthesia. After buccal and lingual intracrevicular incisions, full-thickness mucoperiosteal flaps were raised. All the granu-

lation tissue was removed from the defects, and the roots were thoroughly scaled and planed using hand and ultrasonic instruments. The surgical sites were rinsed with sterile saline.

PerioGlas (US Biomaterials, Alachua, FL, USA) was used as a bioactive glass graft material in the present study. PerioGlas was mixed with sterile saline to form a paste according to the manufacturer's instructions and placed over the defects in BG group. Atrisorb (Atrix Laboratories, Fort Collins, CO, USA), an absorbable polylactide membrane made of polylactic acid, was used for GTR. Atrisorb was also prepared according to the manufacturer's instructions and placed over the defects in BM group. Flaps were replaced and secured by a 4–0 silk suture utilizing an interrupted suture technique to achieve primary closure. Patients were prescribed a 0.2% chlorhexidine gluconate mouthrinse to be used twice a day for 2 weeks. Sutures were removed 1 week after surgery. Recall appointments for supragingival professional tooth cleaning and oral hygiene reinforcement were scheduled every second week during the observation period.

Histopathological analysis

Gingival tissue samples of the patients in both groups were collected at periodontal surgery (baseline) and at 12 weeks after surgery. All surgical procedures were performed at a specified time in the morning to avoid possible cyclical variations of the proliferative index, and to maintain uniform fixation and tissue processing. The biopsies of the gingival papilla were taken from the area of the intrabony defect at the buccal aspect of the gingiva before raising the flap (Fig. 1). The pre- and postbiopsies (Fig. 2) were taken from the same site carefully without leaving the marginal bone exposed. Also, no marginal bone was included at all. Then, these samples were fixed in 10% neutral buffered formalin, embedded into paraffin, and serial sections of samples were obtained in the bucco-lingual direction at 4 μm-thickness. The sections were used for hematoxylin and eosin (H&E) and immunohistochemical staining. In the H&E-stained sections, tissue inflammation was evaluated by counting the inflammatory cells (macrophages and lymphocytes) in a 36-μm^2 area including epithelium and the underlying connective tissue (Fig. 3) by a light microscope (BH2 Research Microscope, Olympus, Tokyo, Japan) transferred to a monitor with a camera apparatus (Objective ×3.3, F10 CCD Camera, Panasonic, Osaka, Japan) at a magnification of ×4,165 [8].

Immunohistochemical analysis

The sections, used for immunohistochemical staining, were taken over poly-1-lysine coated lams, dried at room

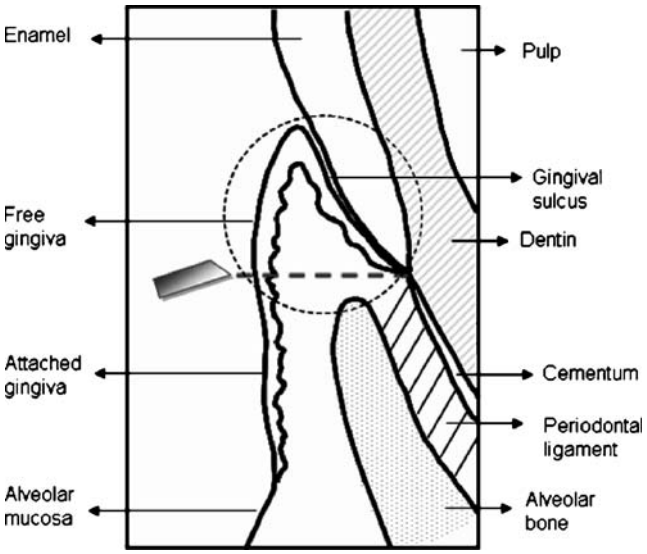

Fig. 1 Schematic illustration of the place and shape of the biopsy. The *interrupted line* indicates the bottom of the biopsy

temperature, deparafinized, and washed with distilled water. PCNA was demonstrated using an improved biotin–streptavidin immunoperoxidase technique according to the manufacturer's protocol (Universal Immunostaining Kit, DBS 1020 Serpentine Lane, # 111, Pleasanton, CA, 94566, USA). To reduce the nonspecific disclosing due to endogenous peroxidase, sections were washed twice with phosphate buffered solution (PBS, pH 7.2), incubated in 3% H_2O_2 (10–15 min), and protein blocked (Goat DAKO, CA, USA) (5 min) at room temperature. The sections were then incubated for 30 min at room temperature with primary monoclonal anti-PCNA antibody (Mouse, monoclonal Clone: PC10, DAKO, CA, USA), washed four times with PBS, and stored in biotinylated secondary antibody (anti-mouse & rabbit) for 20 min. After washing with PBS, the slides were incubated for 20 min in streptavidin conjugated peroxidase solution. After washing four times with PBS, diaminobenzidine (DAB) was added for color

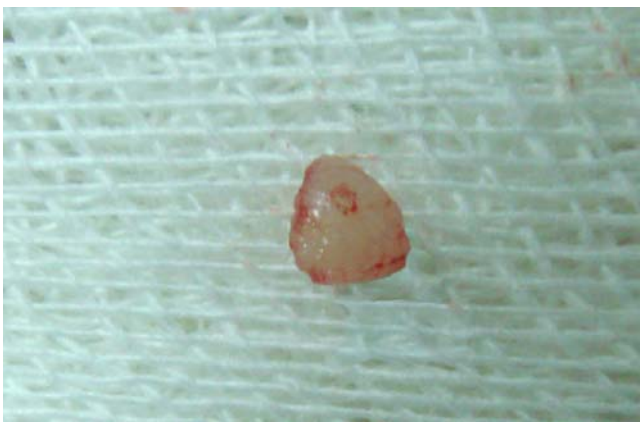

Fig. 2 Clinical photograph of the biopsy sample which was taken immediately after the procedure

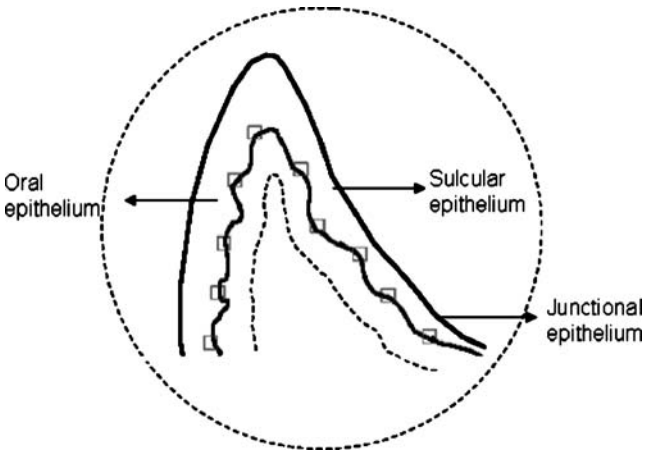

Fig. 3 Schematic illustration of the areas selected for the counting of inflammatory cells and proliferative cells. The inflammatory cells were counted in the epithelium and connective tissue above the *interrupted line*. The proliferative cells were counted in 10 microscopic fields indicated with *boxes*

development (3–5 min) and rinsed with distilled water. After washing for 2 min under tap water, the sections were lightly counterstained with Mayer's hematoxylin (1 min) and washed in running water. They were stored 10 min in alcohol and 3 min in xylene and then mounted in synthetic resin.

In each section, more than 1,000 epithelial cells were counted in 10 microscopic fields selected in the basal layer of the oral, sulcular, and junctional epithelium with ×400 magnification (Fig. 3). Every nucleus with brown color was considered to be positive for PCNA regardless of staining intensity. PCNA-proliferative index which was expressed as the percentage of PCNA+ cells per total number of nucleated epithelial cells counted was used as the indicator of proliferative activity [8].

Statistical analysis

The Shapiro–Wilk test was used to investigate whether the data were normally distributed or not. Wilcoxon nonparametric test was used for statistical comparisons between preoperative and postoperative 12-week measurements of PPD, CAL, PI, GI, and number of the inflammatory cells in each of the treatment modalities. Intergroup differences in these parameters between BG group and BM group were statistically evaluated by Mann Whitney *U* nonparametric test. For the statistical analysis of PPD and CAL, only the recordings representing the deepest clinical site in each defect were used [32].

Intragroup comparisons in PCNA-proliferative index between baseline and postoperative 12 weeks measurements were performed by paired *t* parametric test. Intergroup differences between BG group and BM group were statistically evaluated by Student *t* parametric test.

The statistical analysis was performed using a commercially available software program (SPSS version 12.0, SPSS, Chicago, IL, USA). Data are shown as mean± standard error of means. Significant levels were calculated for $p<0.05$.

Results

Clinical findings

The comparison of preoperative clinical parameters revealed no significant difference between the two groups ($p>0.05$). Intragroup comparisons showed that there was a significant decrease in PPD and gain in CAL over preoperative findings in both groups at postoperative 12 weeks ($p<0.01$). The changes in PPD were 4.40± 0.22 mm in BG group and 4.50±0.17 mm in BM group. The preoperative CAL was found to be improved by an average of 3.60±0.27 mm in BG group and 3.80±0.25 mm in BM group. GI scores were also decreased at postoperative 12 weeks compared to the preoperative data ($p<0.01$), whereas PI scores were not different from the preoperative values in both two groups ($p>0.05$). Intergroup comparisons of postoperative data showed that there was no significant difference between two groups ($p>0.05$). The clinical findings are summarized in Table 1.

Histopathological findings

Remarkable decrease in inflammatory cell infiltration was observed at postoperative 12 weeks compared to the baseline data in both treatment modalities (Fig. 4a–d). Intragroup comparisons of the measurements confirmed

Table 1 Preoperative and postoperative clinical findings of the treatment groups

	BG group[a]		BM group[a]	
	Preoperative	Postoperative	Preoperative	Postoperative
PPD (mm)	7.50±0.37	3.10±0.23[b]	7.80±0.33	3.30±0.21[b]
CAL (mm)	7.90±0.31	4.30±0.26[b]	8.40±0.31	4.60±0.22[b]
PI	0.66±0.03	0.63±0.02[c]	0.69±0.03	0.64±0.02[c]
GI	1.39±0.04	0.38±0.03[b]	1.44±0.03	0.43±0.04[b]

Numbers are the means±standard error of means (Wilcoxon and Mann Whitney *U* nonparametric tests).

PPD Probing pocket depth, *CAL* clinical attachment level, *PI* plaque index, *GI* gingival index

[a] No significant difference at preoperative and postoperative values between two groups ($p>0.05$)

[b] Significantly different from the preoperative values ($p<0.01$)

[c] No significant difference from the preoperative values ($p>0.05$)

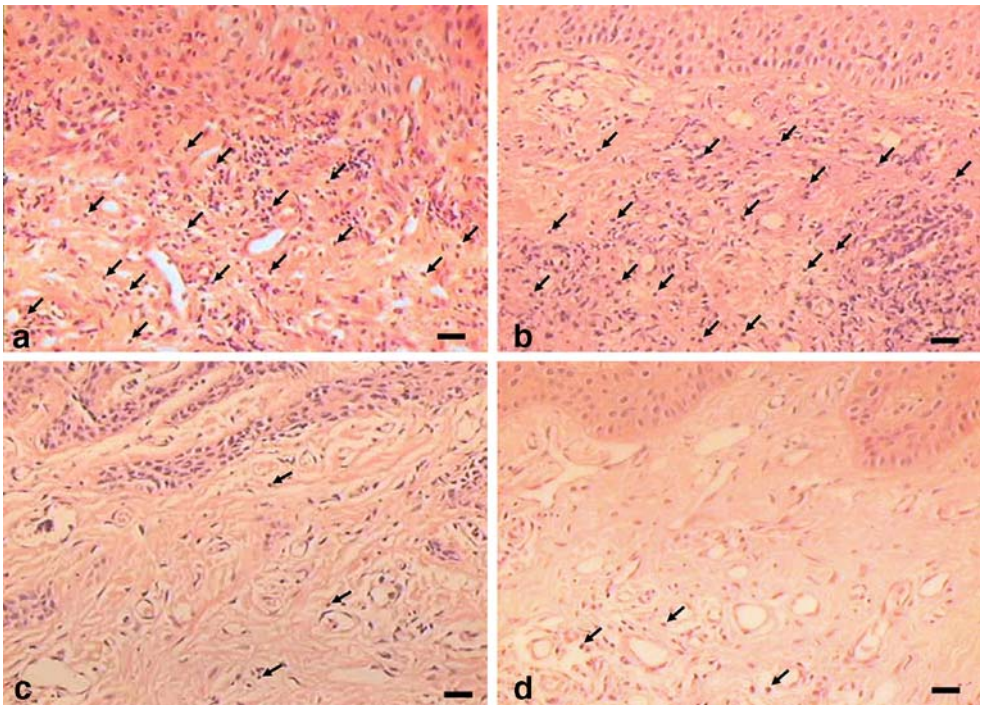

Fig. 4 **a**–**d** Micrographs of the hematoxylin and eosin stained sections showing the distribution of inflammatory cells (macrophages and lymphocytes) which were indicated with *black arrows*. Large areas were occupied with numerous macrophages and lymphocytes indicating higher inflammatory cell infiltration at the baseline in BG group (**a**) and in BM group (**b**). The number of the macrophages and lymphocytes were decreased after 12 weeks in BG group (**c**) and BM group (**d**) compared to the baseline. *Bar* 25 μm

these observations that there was a significant decrease in postoperative number of the inflammatory cells in two groups ($p<0.01$), while intergroup analysis demonstrated that there were no significant differences in both baseline and postoperative number of inflammatory cells between the two groups ($p>0.05$) as shown in Table 2.

Immunohistochemical findings

PCNA staining was confined to the cell nuclei. Although some cells stained more intensely than others, all cells with clear brown nuclei were regarded as positive. PCNA+ cells were distributed heterogeneously throughout the basal cell layer at baseline and at postoperative 12 weeks in both treatment modalities (Fig. 5a–d).

Postoperative scores of PCNA-proliferative index were significantly increased in both groups compared to the baseline scores ($p<0.001$). The increase in PCNA+ cells at postoperative 12 weeks in BG group was significantly higher than those in BM group ($p<0.001$), while there was no significant difference in baseline scores between the two groups ($p>0.05$) as shown in Table 3.

Table 2 The number of inflammatory cells (macrophages + lymphocytes) at baseline and at postoperative 12 weeks in two treatment groups

	Baseline	Postoperative	*p*
BG group[a]	3.30±0.30	1.20±0.25	<0.01
BM group[a]	3.50±0.34	1.00±0.26	<0.01

Numbers are the means±standard error of means (Wilcoxon and Mann Whitney *U* nonparametric tests).

[a] No significant difference at baseline and postoperative values between two groups ($p>0.05$)

Discussion

The results of the present study showed clinical improvement after surgical therapies with both bioactive glass graft material and bioabsorbable membrane with regard to the clinical parameters. No significant differences in any of these clinical parameters were found between two treatment modalities. Several types of graft materials and barrier membranes have been used in surgical treatments with the goal of periodontal regeneration; the highest success in attaining periodontal regeneration is reported to be achieved using absorbable and/or nonabsorbable membranes of various origins [16, 18, 31]. Clinical studies have demonstrated that gains in clinical attachment, decrease in probing depths, and radiographic fill in osseos defects after treatment with bioactive glass alloplastic graft material were not significantly different from guided tissue regeneration [28, 45]. Likewise, our clinical data indicating highly significant decrease in PPD and improvement in CAL after surgical therapies with bioactive glass as well as guided tissue regeneration were in accordance with these reports.

Reduction in probing depth and gain in clinical attachment are the important clinical outcomes of a periodontal regenerative therapy which might have been the results of healing with a new attachment or, alterna-

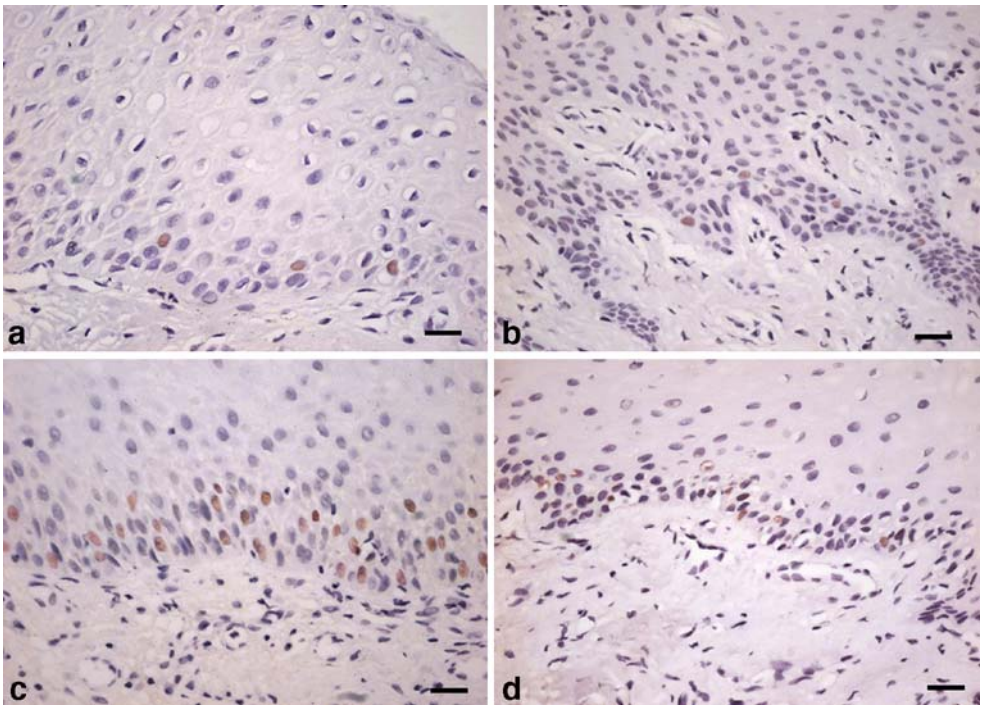

Fig. 5 **a**–**d** Micrographs of the immunohistochemically stained sections showing the distribution of PCNA+ cells. A few PCNA+ cells were observed at the baseline in BG group (**a**) and BM group (**b**). The number of PCNA+ cells was increased at postoperative 12 weeks in the treatment groups; more remarkable in BG group (**c**) compared to BM group (**d**). *Bar* 25 μm

tively, of healing with a long junctional epithelium [21]. It is known that the cells repopulating the root surface after periodontal surgery determine the nature of healing that will form [27]. Periodontal regeneration, the main goal of periodontal surgery, is based on the selective proliferation of cells originating from the periodontal ligament and bone while preventing the proliferation and migration of basal epithelial cells of gingival epithelium [28]. Preclinical studies in nonhuman primates have shown that the use of bioactive glass induces a significant increase in newly formed cementum and attachment, and also prevents apically directed growth of the junctional gingival epithelium [10, 17, 43]. However, in another study, histological analysis of human periodontal defects treated with bioactive glass has revealed healing by a long junctional epithelium with minimal new connective tissue attachment and minimal new bone formation [31]. It is now clear that neither radiological nor clinical improvement of probing depth and attachment level ensures that the true periodontal regeneration has been achieved [1]. Histological analysis is also necessary to evaluate the effectiveness of a regenerative material as well as observing clinical and radiological results. In the light of this information, the present study was designed to determine the proliferative activity of epithelium after surgical treatments with bioactive glass and bioabsorbable membrane to compare the success of bioactive glass graft material with guided tissue regeneration in preventing epithelial cell proliferation.

Table 3 PCNA-proliferative index scores at baseline and at postoperative 12 weeks in two treatment groups

	Baseline	Postoperative	*p*
BG group	13.20±0.58[a]	28.76±0.85l[b]	<0.001
BM group	11.64±0.62[a]	18.36±0.84[b]	<0.001

Numbers are the means±standard error of means (paired *t* and Student *t* tests parametric tests).

[a] No significant difference at baseline values between two groups ($p>0.05$)

[b] Significant difference at postoperative values between two groups ($p<0.001$)

It has been suggested that the long junctional epithelium at the early stage of the periodontal healing possesses remarkably high proliferative activity in supplying epithelial cells, so that long junctional epithelium was able to become established on the exposed root surface after periodontal surgery [42]. Because the cells of junctional epithelium are known to exhibit a higher turnover rate than the cells within other regions of gingival epithelium and many of them are involved in the proliferating cell cycle, proliferating activity of the epithelium after surgical therapies is closely associated with the formation of the long junctional epithelium [9, 40]. It is known that increased proliferative activity of epithelium is also related to the gingival inflammation [8]. However, in the present study, gingival index scores and number of the inflammatory cells, indicators of gingival inflammation, were decreased while PCNA-proliferative index scores were

increased in the epithelium after 12 weeks. In light of these observations, it is relevant to assume that increased proliferative activity is more associated with the formation of a long junctional epithelium rather than the gingival inflammation in this study.

As far as we know, there has been no study comparing the proliferative activity of epithelium after periodontal surgical therapies. In the past, counting the cells in the mitotic phase was the only method available to assess proliferative activity in human gingival epithelium [24, 33]. In recent years, nuclear antigens in proliferating cells have been identified [23, 41, 42]. PCNA, one of the nuclear antigens, has been found in the proliferative component of the normal tissues [15]. In our study, PCNA immunolocalization was used to determine the proliferative activity of gingival epithelium after surgical treatments. It was observed that PCNA+ cells were distributed heterogeneously throughout the basal cell layer at baseline and at postoperative 12 weeks in both treatment modalities. Such a result is also supported by our previous study, in which PCNA expression was found to be localized mainly in the basal layers of epithelium in cyclosporine A-induced gingival overgrowth [8]. Classic literature on the subject also appears to favor the notion that proliferation of gingival epithelial cells takes place by mitosis in the basal layer and less frequently in the suprabasal layers [5].

In an experimental study in which long junctional epithelium is created by inserting a rubber piece between the molar teeth, it has been reported that the long junctional epithelium becomes established in 4 weeks and becomes shorter at 12–28 post weeks [42]. After 12 weeks, a distinct connective tissue attachment associated with the newly formed cementum and thick collagen fibers has been discernible by the shortening and movement of the junctional epithelium [42]. In the present study, the proliferative activity of epithelium after 12 weeks increased after surgical treatments with both bioactive glass and bioabsorbable membrane compared to the baseline data. This increase was significantly higher in BG group compared to those in BM group indicating the epithelial proliferative activity was more prominent after surgical treatment with bioactive glass graft material. No significant difference was found in baseline proliferative activity between two groups probably associated with the split mouth design.

To the best of our knowledge, this is the first report comparing the proliferative activity of epithelium after surgical treatment of intrabony defects with bioactive glass and bioabsorbable membrane. In one study comparing only the clinical and radiological effectiveness of bioactive glass and bioresorbable membrane, it has been shown that both two materials can be used equally successfully in the treatment of intrabony defects [28]. In the present study, despite the evidence of similar clinical improvements after both treatment modalities, our immunohistochemical analysis demonstrated differences in healing activities of the cells between two treatments; higher epithelial cell proliferation after treatment with bioactive glass compared to bioabsorbable membrane.

Conclusions

Within the limitations of the period and number of the study population, our data suggested that proliferation of gingival epithelial cells is more prominent after treatment of intrabony defects with bioactive glass compared to the treatment with bioabsorbable membrane. Based on the present data, it is important to note that determining the epithelial proliferative activity with certain nuclear antigens can be used as an indicator to evaluate the type of healing after periodontal surgical treatments. Additional studies with shorter and longer periods of evaluation should be undertaken to further strengthen the assumptions of the present study about the proliferative activity of gingival epithelium after surgical treatments of intrabony defects with bioactive glass graft materials and bioabsorbable barrier membranes.

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