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Antiproliferative effect of topic hyaluronic acid gel. Study in gingival biopsies of patients with periodontal disease

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Summary. Hyaluronic acid (HA) is the most abundant glycosaminoglycan of high molecular weight in the extracellular matrix of soft periodontal tissues. Our group recently demonstrated an HA-induced reduction in lymphoplasmocyte inflammatory infiltrate in periodontal disease.

The objective of this study was to determine the effect of an HA gel of high molecular weight on cell proliferation, inflammation, and different periodontal lesion parameters.

A double-blind clinical trial was conducted on the effect of an HA gel on cell proliferation in gingival biopsies from 28 patients with periodontal disease. A split-mouth design was used, randomly applying the gel to one quadrant and a placebo to the contralateral one. A gingival biopsy was taken for histopathological and immunohistochemical study, in order to determine the expression of cell proliferation antigen Ki-67 and to evaluate the inflammatory infiltrate.

HA gel treatment induced a significant reduction in the proliferation index of the gingival epithelium, with 276 (range 234-317) Ki-67-positive cells per mm^2 in treated samples versus 514 (range 158-876) per mm^2 in controls (Mann-Whitney U test, $p<0.003$). In 13 patients, the number of Ki-67-positive fibroblastic cells was reduced by the treatment, whereas in 6 patients no differences were found (global difference, $p=0.12$). In 10 patients, Ki-67-positive cells were decreased in chronic inflammatory infiltrate present in the lamina propria, whereas in 6 patients no differences were found (global difference, $p=0.054$).

We conclude that high molecular-weight HA gel reduces cell proliferation in epithelial cells such as fibroblasts and lymphocytes, abates the inflammatory process, and improves the periodontal lesion in patients with chronic periodontitis.

Key words: Hyaluronan, Ki-67, periodontal disease

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Introduction

Hyaluronic acid (HA) is a large nonsulfated glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and N-acetyl-glucosamine and is a major component of the extracellular matrix of many tissues (Toole et al., 1989).

HA is widely sequestered in the extracellular matrix and is secreted by many cells, especially fibroblasts. It plays an active role in regulating cell behavior, including random motility, chemotaxis, invasion, proliferation, shape, and metabolic reactions (Laurent and Fraser, 1992). HA appears to have paradoxical effects on cell behavior. HA synthesis has been shown to increase during cell migration (Toole, 1972, 1991), mitosis (Brecht et al., 1986) and tumor invasion (Toole et al., 1979), whereas other authors have demonstrated that HA promotes cell adhesion (Erickson and Turley, 1983; Miyake et al., 1990) and cell-cell aggregation (Underhill, 1982) and reduces proliferation (Goldberg and Toole, 1987; West and Kumar, 1989).

HA participates in tissue repair and wound healing and is used topically as an anti-inflammatory and antiedematous agent (Häkkinen et al, 1993; Oksala et al, 1995). HA has been used in the healing of incisions in maxillofacial surgery (Berrone et al., 1992), in dentin repair and pulpal regeneration (Sasaki and Kawamata-kido, 1995), and in patients with periodontal disease, especially gingivitis (Brandimarte, 1968). Recently, our group showed that HA treatment reduces the severity of gingival inflammatory infiltrate in periodontal patients (Mesa et al., 2001).

The objective of the study was to determine the effect of an HA gel of high molecular weight on cell proliferation, inflammation, and different periodontal lesion parameters.

Materials and methods

Twenty-eight patients with different degrees of periodontitis who had received no dental treatment during the previous 6 months were randomly selected from the database of our center. All patients gave their

written informed consent to participate in the clinical trial, which was approved by the Ethics Committee of our institution.

The criteria for inclusion in the study were: previous treatment at our center; age over 18 years; clinical history of periodontal disease; no current intake of anti-inflammatory or antibiotic drugs, and the patient's informed consent to enter the study. Patients who did not meet any of these criteria or who failed to complete the treatment correctly were excluded from the study. Four patients were excluded for receiving anti-inflammatory treatment during the study period, two for failing to correctly complete the drug and placebo treatment under study, and one for missing the second examination at one month. Thus the final study group consisted of 21 patients.

A split mouth design was used for this double-blind clinical trial, dividing either the maxilla or mandible, according to the number of teeth (arch with most teeth was selected), into two quadrants. At the beginning and end of the study, the mesial, distal, buccal and lingual aspects of a minimum of six teeth in each quadrant were evaluated using a Marquis probe (marking at 3 mm intervals with color codes). At the same examinations, the Ainamo and Bay (1975) gingival bleeding index was measured. The quadrant to receive the hyaluronic acid gel (test) was selected randomly for each patient and the contralateral site was designated to receive the placebo (control). The high molecular-weight HA (Gengigel, ICN HUBBER, Barcelona, España) comprises sodium hyaluronate and excipients (cellulose, water, xylitol, hydrogenated castor oil, polyvinyl alcohol, polycarbophil, aroma, and sodium hydroxide [CI 42090]). The test and control gels were applied topically with the finger, twice a day after tooth-brushing, on the entire quadrant on buccal and lingual surfaces. At the beginning of the study, all patients were instructed how to maintain a correct oral hygiene. After one month of treatment with the gel, the patients were re-examined.

A biopsy of the gingival papilla was performed at the buccal aspect between the second premolar and first molar in each quadrant for histopathological study. Immediately after their extraction, the specimens were fixed in 10% buffered formalin, embedded in paraffin, and 3 μm sections were cut for hematoxylin-eosin staining. The grading of the inflammatory infiltrate was established by randomly selecting and counting 10 fields of lamina propria at x400 magnification. Inflammatory infiltrate was considered "absent" when none of the 10 fields showed inflammatory cells; "slight" when at least 5 fields contained inflammatory cells that occupied <50% of the field; "moderate" when at least 5 fields showed inflammatory cells that occupied >50% of the field; and "intense" when all 10 fields evaluated showed inflammatory cells that occupied >90% of the field.

Immunohistochemical techniques were used to study the expression of the proliferation antigen Ki-67. The histological sections were dewaxed, hydrated, and heat-treated under pressure for antigenic unmasking. The incubation with the anti-Ki-67 antibody (MIB1 clone, Dako, Barcelona, Spain) was maintained at 1:80 dilution for 60 min, using the streptavidin-biotin-phosphatase alkaline method (Master Diagnóstica, Granada, Spain) in an automatic immunostainer (Labvision, Fremont CA, USA). A count was made of the number of nuclei positive for the antibody in the gingival epithelium, in fibroblasts of the lamina propria and in the inflammatory infiltrate (15 fields at x400 magnifications were assessed). The results were expressed as number of positive cells per mm^2.

Statistical analysis

The Shapiro-Wilk and Levene tests were used to test the normality and homocedasticity of the variables, respectively. After the descriptive analysis, a stratified bivariate analysis was performed to determine associations between the study variables, using the Wilcoxon signed-ranks test, the McNemar test, the Mann-Whitney U non-parametric test and the Spearman correlation. The samples were stratified into control and test. The confidence interval was 95% ($p<0.05$). The statistical analysis was performed using the SPSS-Windows 9.0 program (SPSS Inc, Chicago, USA).

Results

The mean age of the patients was 44.5 years (CI 95% 38.22-52.69, s=10.77). Thirteen patients were female (61.9%) and eight male (38.1%).

The gingival sections presented chronic inflammatory infiltrate of variable intensity, mainly composed of patches of lymphocytes, plasma cells and macrophages in the lamina propria, frequently surrounding vascular structures. The patients with more intense inflammatory infiltrates presented exocytosis towards the mucosal epithelium. There was a significant association between the quadrants that received HA treatment and absence of inflammatory infiltrate (corrected typified residue = 2.9) at the one-month follow-up. Although there was no significant association between the treatment and other grades of intensity, 61.9% of control specimens showed moderate-severe inflammatory infiltrate at the end of the study, whereas only 28.4% of the test specimens showed this intensity of infiltrate (Table 1). The HA treatment produced a

Table 1. Results of the evaluation of the inflammatory infiltrate in the gingival biopsies at 30 days after treatment.

	INFLAMMATORY INFILTRATE				
	Absent*	Slight	Moderate	Intense	Total biopsies
Control	1 (4.7%)	7 (33.3%)	5 (23.8%)	8 (38.1%)	21
Test	9 (42.8%)	6 (28.5%)	3 (14.2%)	3 (14.2%)	21
Total	10 (23.8%)	13 (30.9%)	8 (38.1%)	11 (26.2%)	42

*Pearson's chi-squared = 9.419; Monte Carlo significance =0.027; C.I.99%=[0.022-0.029]

reduction of inflammatory infiltrate in 65% of the patients (Fig. 1A,B).

Parallel to the reduction of inflammatory infiltrate, a significant decrease in bleeding was recorded at the end of the study. In the test quadrants, the number of bleeding teeth was significantly reduced from 42 (68.85%) at the beginning of the study to 31 (50.8%) at the end (p=0.019). In the control quadrants, although 8 of the 21 showed a higher bleeding index, there was also an overall reduction in the number of teeth with gingival bleeding (McNemar test, p=0.004).

In the gingival epithelium, the HA significantly reduced the proliferation index in the test quadrants from a mean of 514 Ki-67-positive cells per mm^2 (range 158-876) to a mean of 276 per mm^2 (range 141-353) (Mann Whitney U test, p<0.003) (Table 2) (Fig. 2A, B). In 10 patients, the proliferative (Ki-67-positive) cells decreased in the chronic inflammatory infiltrate present in the lamina propria of the test quadrant, whereas in 6 patients the immunostaining was similar; the overall difference was very close to significance (Mann-Whitney test, p=0.054) (Fig. 3A,C). In 13 patients, the number of Ki-67-positive fibroblastic cells decreased in the HA-treated quadrant, whereas 6 patients showed no change; the overall difference was again close to significance (Mann Whitney U test, p=0.123) (Fig. 3B, D). No consistently significant correlation was found between the expression of Ki-67 in the different cell types and the age of the patients or the periodontal parameters under study, except for a direct relationship between fibroblastic proliferation and periodontal lesion (probe depth) (Table 3).

The beneficial effect of HA treatment on the progression of the lesions was shown by the evolution of the periodontal pocket depth. The initial depth of periodontal pockets in the control and test quadrants was very similar (2.84±0.94 mm vs. 2.71±0.85 mm, respectively). Comparison between these measurements

Table 2. Comparison between groups of immunohistochemical expression of Ki-67 in different cells at 30 days after treatment.

CELLS	Ki-67/mm^2	CONFIDENCE INTERVAL FOR THE MEAN AT 95%		MW-U	P VALUE
		Lower level	Upper level		
Epithelium					
Control	514.2±62.2*	375.4	653.0		
Test	276.1±18.4	234.8	317.2	17	P=0.003
Fibroblast					
Control	3.22±0.86	1.28	5.16		
Test	1.46±0.75	-0.22	3.15	31.5	P=0.054
Inflammatory Infiltrate					
Control	12.0±6.24	-1.88	25.9		
Test	4.99±2.96	-1.61	11.58	37.5	P=0.12

MW-U: Mann-Whitney U test. * mean ± standard deviation.

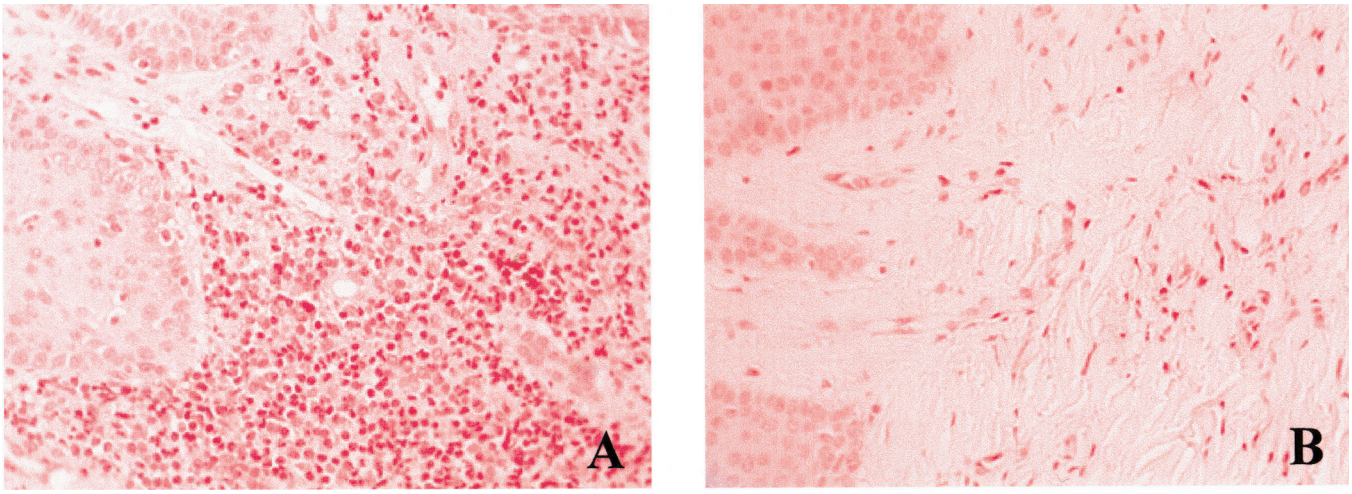

Fig. 1. A. Gingival biopsy of buccal zone between second premolar and first molar after 30 days of placebo treatment. Characteristic morphological substrate of chronic periodontitis: gingival mucosa with abundant inflammatory infiltrate localized in the lamina propria, distributed in perivascular accumulations and composed of lymphocytes, mature plasma cells, and macrophages. Hematoxylin-eosin, x 100. **B.** Gingival biopsy of buccal zone between second premolar and first molar. Histopathological changes in the gingiva contralateral to that in Figure 1A after 30 days of treatment with Gengigel®. Major reduction in inflammatory infiltrate. Hematoxylin-eosin, x 100

(measured at four points per tooth) at the beginning and end of the study showed a significant progression in the control quadrants (3.05±0.94 mm, Wilcoxon test: Z -2.491, Monte Carlo test, p=0.01), whereas there was no change in the test quadrants (2.71±0.82 mm, Wilcoxon test: Z -0.561, Monte Carlo test, p=0.622).

Discussion

HA is a glycosaminoglycan of high molecular weight of the extracellular matrix involved in growth and inflammation/repair. It supplies hydration and elastic properties to the skin and mucosas, including the gingival mucosa. The identification of new receptors and binding proteins and the elucidation of HA-dependent signaling pathways are providing novel insights into the true biological functions of this intriguing molecule (Lee and Spicer, 2000).

The effects of HA are molecular weight- and concentration- dependent and is cell-specific, being influenced by the type of receptor expressed. Its apparently paradoxical effect is markedly dependent on the molecular weight and concentration of the HA (Goldberg and Toole, 1987). Slevin et al. (1998) showed that oligosaccharides of HA composed of 3 to 10 disaccharides (o-HA) induce tyrosine phosphorylation of multiple proteins and produce proliferation in endothelial cells but that the native high molecular-weight HA (n-HA) has little effect. The o-HAs increase the tyrosinase kinase activity, resulting in the activation of specific isoforms of protein kinase C and then of the cytoplasmic enzyme cascade (Raf-1 kinase, MAP kinase kinase [MEK-1], and mitogen-activated kinase [ERK-1]) with stimulation of early response genes and cell proliferation (Slevin et al., 1998). The present findings that n-HA decreased proliferation in epithelial, fibroblastic and leukocyte cells may be explained by the high molecular weight of the molecular used, consistent with the above mechanism.

The ability of HA to penetrate the skin when applied topically is also controversial. Brown et al. (1999) applied ^{3}H-labeled HA gel on intact skin in mice and observed cellular uptake in epidermis, dermis and in lymphatic endothelium, using chromatographic analysis to demonstrate HA absorption. On the other hand, Laugier et al. (2000) reported that HA does not penetrate the stratum corneum of the skin. No studies have been published on the ability of HA to penetrate the gingival mucosa and the gingival sulcus, which have no stratum corneum.

Periodontal inflammation is characterized by the accumulation of inflammatory cells in connective-

Table 3. Spearman correlation coefficient between Ki-67 expression and clinical variables at end of study.

Ki-67-POSITIVE CELLS	GROUP	AGE	BLEEDING	PROBING DEPTH
Epithelium	Control	0.178 (0.601)*	-0.377 (0.283)	-1.730 (0.612)
Test		-0.169 (0.620)	-0.232 (0.519)	-0.091 (0.790)
Fibroblast	Control	0.415 (0.205)	-0.059 (0.872)	0.584 (0.059)
Test		0.527 (0.095)	0.326 (0.358)	0.657 (0.028)
Inflammatory Infiltrate	Control	0.037 (0.914)	0.067 (0.854)	0.055 (0.872)
Test		0.596 (0.053)	0.022 (0.952)	0.440 (0.176)

*Spearman Rho coefficient; with level of bilateral significance in parentheses.

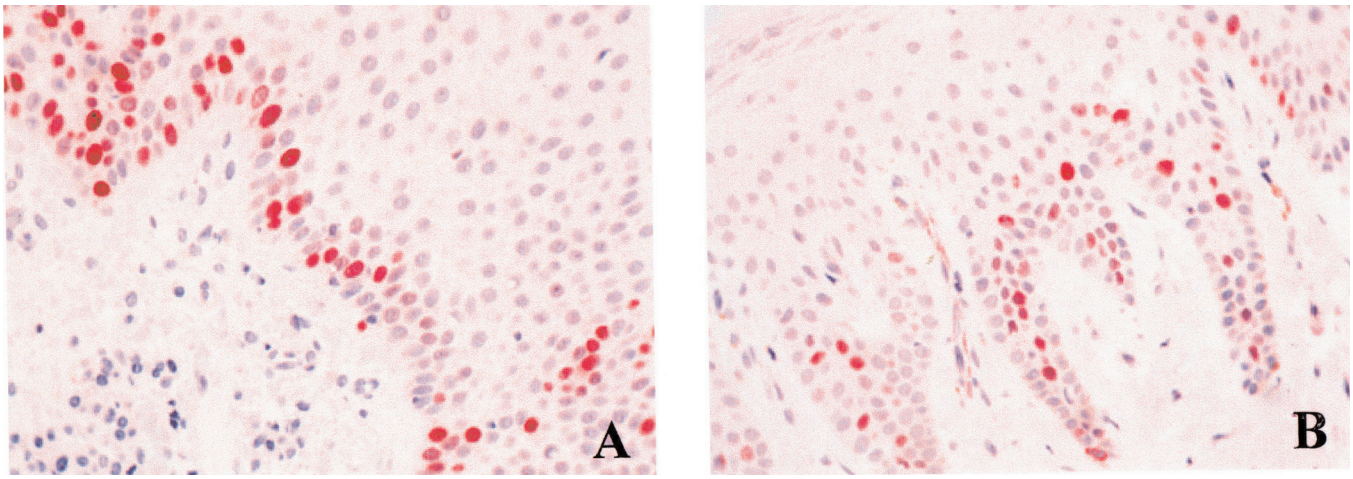

Fig. 2. A. Nuclear expression of proliferation antigen Ki-67 in gingival biopsy of buccal zone after 30 days of placebo treatment. Streptavidin-biotin-phosphatase alkaline, x 200. **B.** Nuclear expression of proliferation antigen Ki-67 in gingival biopsy of contralateral buccal zone after 30 days of Gengigel® treatment. Major reduction in number of immunostained nuclei. Streptavidin-biotin-phosphatase alkaline, x 200

vascular tissue. Different mechanisms have been proposed to explain the effect of HA on the inflammatory process. Cortivo et al. (1986) and Weigel et al. (1986) considered that HA produces a physical barrier against bacteria and their products in the extracellular matrix. Haapasalo et al. (1996) demonstrated that the chemotrypsin-induced binding of HA to *Treponema dentícola*, a bacteria isolated in periodontal lesions, prevented its destructive action on the periodontium. More recently, HA has been shown to be the main ligand of the CD44 receptor (Goodison et al., 1999). Isoforms of the CD44 receptor are involved in the initial binding of leukocytes to endothelial cells activated by inflammatory processes (Johnson et al., 2000) and in the extravasation of lymphocytes from blood vessels (Siegelman et al., 2000). Murakami et al. also ascribed the persistence of lymphocytic inflammatory infiltrate in the lamina propria to the intense binding of the lymphocytes with gingival fibroblasts, mediated by VLA adhesion molecules of the integrin family, LFA1/ICAM-1 and CD44/HA (Murakami et al., 1996, 1997). The significant reduction in inflammatory infiltrate in HA-treated versus placebo-treated quadrants in the present study may be explained by two mechanisms: on one hand, by the reduction in expression of the proliferation antigen Ki-67 in inflammatory cells in the lamina propria, and on the other hand by the blocking of the CD44 receptor by its HA ligand. HA has been shown to act by the latter mechanism in inflammation (Pure and Cuff, 2001) but this issue has yet to be addressed in periodontal disease. Future prospects for the treatment of inflammation

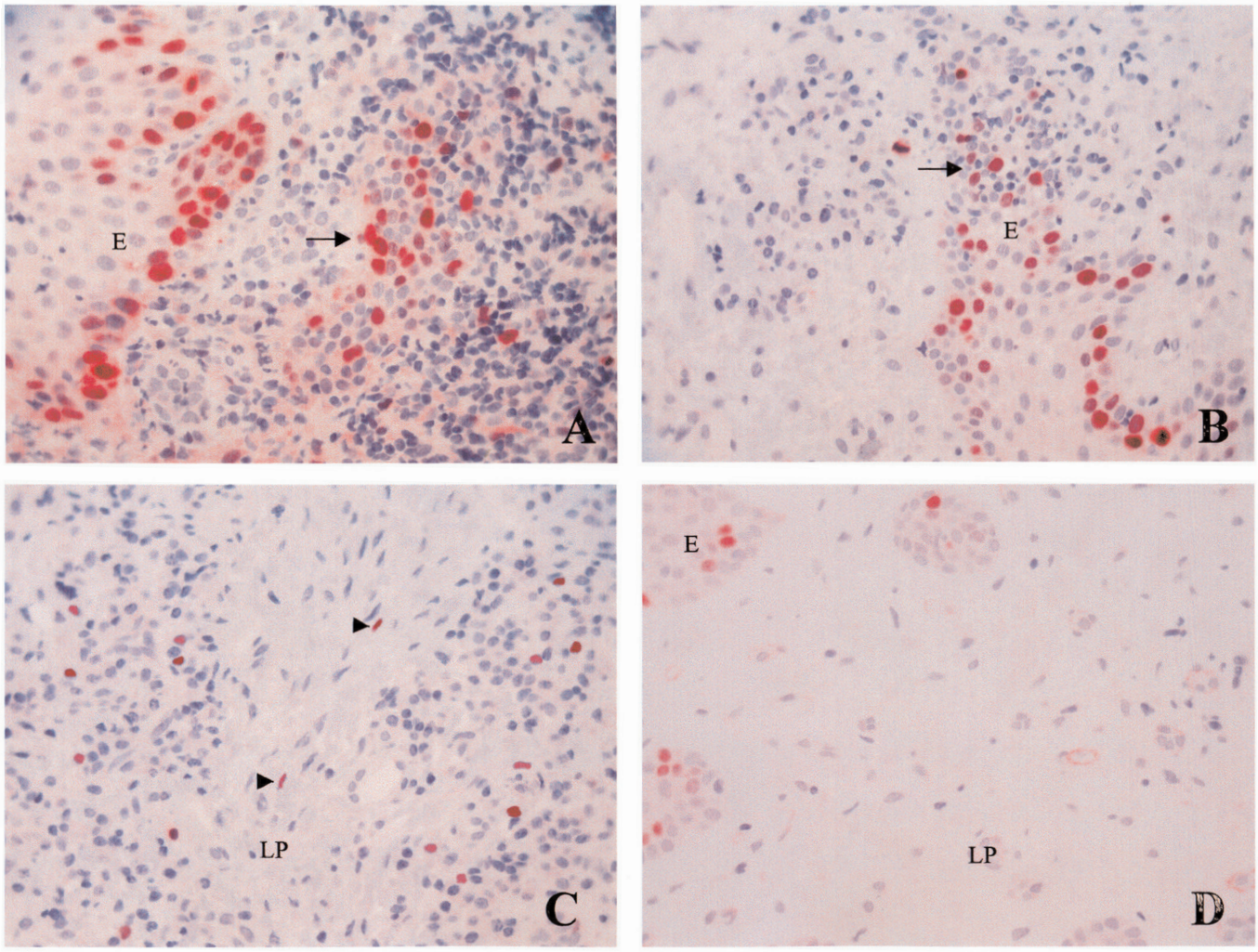

Fig. 3. A and C. Gingival biopsy: Nuclear expression of proliferation antigen Ki-67 in inflammatory infiltrate (arrow) **(A)** and fibroblastic cells (arrow head) (C) of buccal zone after 30 days of placebo treatment. E: Epithelium. LP: Lamina propria. Streptavidin-biotin-phosphatase alkaline, x 200. **B and D.** Gingival biopsy: Nuclear expression of proliferation antigen Ki-67 in inflammatory infiltrate and fibroblastic cells of contralateral buccal zone after 30 days of Gengigel® treatment. Note major reduction in number of inflammatory cells and immunostained nuclei (arrow) **(B)** and the absence of inflammatory infiltrate and immunostaining of fibroblastic cells. E: Epithelium. LP: Lamina propria. Streptavidin-biotin-phosphatase alkaline, x 200

should therefore include the targeting of specific inflammatory pathways in both immune cells and parenchymal cells. CD44, a cell-adhesion molecule that is ubiquitously expressed on leukocytes and parenchymal cells has been implicated, together with its ligand hyaluronan, in several inflammatory diseases. The mechanisms of action of CD44-HA interactions in inflammation might provide potential targets for therapy (Pure and Cuff, 2001).

Pagnacco et al. (1997) investigated marginal gingivitis using a similar design to the present study and demonstrated that HA had a significant preventive and therapeutic effect in improving the reddening, bleeding and inflammation of the gingival mucosa. In the present study, both treated and control quadrants presented a significant improvement in gingival bleeding. The improvement in the control gingiva is possibly due to an improved oral hygiene after instruction in the correct brushing technique, because no other dental treatment was carried out and the papilla biopsies showed no significant reduction in inflammation in the control quadrants.

The depth of periodontal pockets is a clinical sign of periodontal inflammation. In the present study, the one-month HA treatment prevented the progression of lesions and even significantly reduced the mean depth on lingual surfaces, whereas there was an increase in all surfaces of the periodontal lesions in the control quadrants, with a significant increase of 0.40 mm in distal surfaces. Although there are scant published studies of the effect of HA on the periodontal pocket (Mesa et al., 2001), the reduced proliferation of the fibroblasts suggests that the improvement is not related to an increased synthesis of extracellular matrix components but rather to the drug's effect on the inflammatory process. It is well known that in chronic inflammation the gingiva enlarges and the gingival margin extends towards the dental crown, increasing the depth of the gingival pocket (Schroeder, 1970). Our biopsy results confirmed this phenomenon, because the inflammatory infiltrate was maintained or even increased in the control quadrants, whereas the test quadrants showed a clear reduction of the inflammatory infiltrate, with a maintained or reduced pocket depth.

We conclude that high molecular-weight HA reduces cell proliferation in epithelial cells such as fibroblasts and lymphocytes, abates the inflammatory process, and improves the periodontal lesion in patients with chronic periodontitis.

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