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Guided tissue regeneration may modulate gene expression in periodontal intrabony defects: a human study

L. L. Lima[1], P. F. Gonçalves[2], E. A. Sallum[1], M. Z. Casati[1], F. H. Nociti Jr[1]

[1]Division of Periodontics, School of Dentistry at Piracicaba, University of Campinas, Piracicaba, SP and [2]Department of Dentistry, Division of Periodontics, School of Biologic and Health Sciences, Federal University of Vales do Jequitinhonha e Mucuri, Diamantina, MG, Brazil

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Background and Objective: Guided tissue regeneration has been shown to lead to periodontal regeneration; however, the mechanisms involved remain to be clarified. The present study was carried out to assess the expression of genes involved in the healing process of periodontal tissues in membrane-protected vs. nonprotected intrabony defects in humans.

Material and Methods: Thirty patients with deep intrabony defects (≥ 5 mm, two or three walls) around teeth that were scheduled for extraction were selected and randomly assigned to receive one of the following treatments: flap surgery alone (control group) or flap surgery plus guided tissue regeneration (expanded polytetrafluorethylene (e-PTFE) membrane) (test group). Twenty-one days later, the newly formed tissue was harvested and quantitatively assessed using the polymerase chain reaction assay for the expression of the following genes: alkaline phosphatase, receptor activator of nuclear factor-κB ligand, osteoprotegerin, osteopontin, osteocalcin, bone sialoprotein, basic fibroblast growth factor, interleukin-1, interleukin4, interleukin-6, matrix metalloproteinase2 and matrix metalloproteinase9.

Results: Data analysis demonstrated that mRNA levels for alkaline phosphatase, receptor activator of nuclear factor-κB ligand, osteoprotegerin, osteopontin, bone sialoprotein, basic fibroblast growth factor, interleukin-1, interleukin-6, matrix metalloproteinase-2 and matrix metalloproteinase -9 were higher in the sites where guided tissue regeneration was applied compared with the control sites ($p < 0.05$), whereas osteocalcin mRNA levels were lower ($p < 0.05$). No difference was observed in interleukin-4 mRNA levels between control and test groups.

Conclusion: Within the limits of this study, it can be concluded that genes are differentially expressed in membrane barrier-led periodontal healing when compared with flap surgery alone, and this may account for the clinical outcome achieved by guided tissue regeneration.

Francisco Humberto Nociti Jr, Faculdade de Odontologia de Piracicaba, University of Campinas, Avenida Limeira 901, Areião, Cep: 13414-903, Piracicaba, SP, Brazil
Tel: +55 19 21065298
Fax: +55 19 21065301
e-mail: nociti@fop.unicamp.br

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Periodontal regeneration is a complex wound-healing process that involves the structural and functional rebuilding of periodontal tissues that have been lost as a consequence of periodontal disease. The underlying principle of guided tissue regeneration is the use of physical barrier membranes to promote the selective repopulation

of the previously diseased root surface by cells capable of reforming the cellular and extracellular components of new periodontal ligament, new cementum and new alveolar bone (1). A number of histological studies have demonstrated that periodontal regeneration can be achieved *in vivo* by guided tissue regeneration (2–5). Several controlled studies in humans have also shown significant improvements in clinical and radiographic parameters following guided tissue regeneration therapy (6–8). However, there is marked variability between these studies, and clinical predictability is still uncertain, reflecting the complexity of periodontal wound healing, which involves the interaction of distinct cellular types (1).

Although the cells involved in periodontal healing, as well as molecular factors expressed in the regenerative microenvironment, are key factors that ultimately determine the clinical outcome, very few studies have investigated periodontal regeneration by guided tissue regeneration with a mechanistic perspective. Immunohistochemical studies have demonstrated that both mesenchymal and epithelial cells are present in the tissues that are adherent to the membrane and in the regenerated soft tissues taken from the healing periodontal defect (3,9). Cultured *in vitro*, these cells appear to be fibroblast-like in morphology and produce extracellular matrix proteins associated with connective tissue, certain proteases and cytokines (9). However, while these guided tissue regeneration-associated cells also formed mineralized nodules *in vitro*, their precise role in periodontal regeneration is still unclear (10). In an attempt to determine the role that cells play in periodontal regeneration, cells harvested from the regenerating tissue, periodontal ligament and gingiva of periodontal defects treated with guided tissue regeneration were isolated, cultured and characterized (11,12). These studies demonstrated that fibroblasts obtained from the regenerating periodontal defects exhibit characteristics consistent with their ability to facilitate periodontal regeneration.

Recently, tissue engineering was proposed as a method to promote regeneration by controlling the interaction of the various periodontal components and delivering specific cell populations to the appropriate periodontal compartment (13). However, before this can be achieved, greater understanding is required with regard to the cellular and molecular mechanisms that are induced during wound healing and regeneration. In particular, further information is required on the cell phenotype and the extracellular environment required to achieve predictable periodontal regeneration.

Thus, in an attempt to understand in greater detail the molecular mechanisms of periodontal healing following guided tissue regeneration in sites previously affected by chronic periodontal disease, the present study assessed the expression of a number of genes reported to play a role in periodontal regeneration. The expression of these genes was investigated in the newly formed tissue under membrane-protected intrabony defects and compared with that of nonprotected defects, following open flap debridement.

Material and methods

Study population

Thirty patients (aged 29–59 years) with one deep intrabony defect (≥ 5 mm, two or three walls) located in anterior or posterior teeth and indicated for extraction, were included in this parallel, single-blind study. All patients were free of systemic complications and were nonsmokers. Patients were instructed as to the character and purpose of the study and signed their informed consent. The Institutional Ethical Committee on Human Research approved the consent and study protocol. Inclusion criteria for the study were: (i) adult patients with chronic periodontitis, defined as multiple sites (at least eight) with a probing depth of ≥ 5 mm, associated with bleeding on probing and bone loss by radiographs; and (ii) a tooth indicated for extraction, with deep intrabony defects (≥ 5 mm, two or three walls, not located at furcation), without endodontic treatment, clinically detectable dental mobility, caries or restorations close to the gingival margin. The exclusion criteria were: (i) pregnant or lactating women; (ii) anti-inflammatory, antibiotic or hormone use for 6 mo before the study; (iii) evidence of systemic modifiers of periodontal disease, such as smoking, osteoporosis, diabetes or the use of drugs that influence periodontal tissues; and (iv) periodontal treatment for 6 mo before the study.

Study design and surgical procedure

Following supragingival instrumentation and the plaque control phase, the defects were surgically assessed. Prior to the surgery, the subjects were randomly assigned to receive only one of the following treatments:

- control group (n = 15 subjects): intrasucular incisions were made and a buccal–lingual mucoperiosteal flap was raised, granulation tissue was removed from the defect and the tooth surface was completely debrided by scaling and root planing.
- test group (n = 15 subjects): intrasucular incisions were made and a buccal–lingual mucoperiosteal flap was raised, granulation tissue was removed from the defect, the tooth surface was completely debrided by scaling and root planing, and a nonresorbable e-PTFE membrane (Regenerative Material, Gore-tex; Gore Associates, Flagstaff, AZ, USA) was positioned covering the defect area and 2–3 mm of adjacent bone. In all sites (control and test), flaps were coronally positioned and sutured (Gore-tex sutures, WL; Gore Associates). Patients were prescribed antibiotic for 7 d (500 mg of amoxicillin q.i.d.) and analgesic medication for 2 d. Chlorhexidine rinses (0.12%) twice daily were prescribed throughout the whole experimental period (21 d), together with the topical application of a 1% chlorhexidine gel. Twenty-one days after surgery, the regenerating tissue formed in the defect area was carefully collected (after membrane removal for the test sites), rinsed with cold sterile saline solution and stored for the assessment of gene expression using the quantitative polymerase chain reaction

(PCR) technique. In order to avoid the inclusion of any gingival-related soft tissue in the biopsy harvested from the nonmembrane-treated sites, the gingival tissue was carefully dissected from the newly formed tissue in the defect area. The tooth was then extracted and postoperative care was conducted routinely; sutures were removed after 7–10 d.

Gene expression analysis

RNA extraction — The regenerating tissue was harvested from the defects, rinsed with cold sterile saline solution and stored in a tube containing RNAlater® (Ambion Inc., Austin, TX, USA) at −70°C for quantitative assessment of the mRNA levels of the following genes: alkaline phosphatase, receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin, osteopontin, osteocalcin, bone sialoprotein, basic fibroblast growth factor, interleukin-1, interleukin-4, interleukin-6, matrix metalloproteinase(MMP)-2 and MMP-9. Total RNA was extracted using the TRIZOL reagent (Gibco BRL, Life Technologies, Rockville, MD, USA), following the manufacturer's recommendation. RNA samples were resuspended in diethylpyrocarbonate-treated water and stored at −70°C, and the RNA concentration was determined from the optical density of the sample using a Biophotometer (Eppendorff AG, Hamburg, Germany).

Quantitative PCR reactions — • Reverse transcription and primer design: total RNA was DNase treated with Turbo DNA-free® (Ambion Inc.) and 1 μg was used for cDNA synthesis. The reaction was carried out using the transcriptor first-strand cDNA synthesis kit (Roche Diagnostic Co., Indianapolis, IN, USA), following the manufacturer's recommendations. Primers were designed using the LIGHTCYCLER® probe design software (Roche Diagnostics GmbH, Mannheim, Germany) and are presented in Table 1.

• Quantitative PCR reactions: the quantitative PCR reactions were performed in the LightCycler® system (Roche Diagnostics GmbH) using the FastStart DNA Master plus SYBR Green kit (Roche Diagnostic Co.). For each run, water was used as a negative control. The reaction product was quantified using the Relative Quantification tool (LIGHTCYCLER® Software 4; Roche Diagnostics GmbH), with glyceraldehyde-3-phosphate dehydrogenase as the reference gene. Experiments were run twice, with comparable results obtained on each occasion.

Table 1. Primer sequences, annealing temperature and GenBank number for each gene

Gene	Primer sequence (5′ → 3′)	Temperature (°C)	GenBank number
GAPDH	GAAGGTGAAGGTCGGAGTC	53.6	NM_002046
	GAAGATGGTGATGGGATTTC	51.4	
OCN	AGCTCAATCCGGACTGT	60.4	NM_199173
	GGAAGAGGAAAGAAGGGTGC	60.8	
ALP	CGGGCACCATGAAGGAAA	60.1	NM_000478
	GGCCAGACCAAAGATAGAGTT	60.4	
BSP	GAGGGCAGAGGAAATACTCAAT	60.5	NM_004967
	ATTCAAAGCCAAGTTCAGAGATGTAAA	60.1	
OPN	AAAGCCAATGATGAGAGCAA	59.6	NM_000582
	ATTTCAGGTGTTTATCTTCTTCCTTAC	60.0	
bFGF	AACATCTCCTAACTTGTTTAAATGTCC	60.0	NM_002006
	ATCCGGTGCTTCCACGA	59.7	
RANKL	CGACATCCCATCTGGTTCC	56.5	AF_019047
	GCTGGTTTTAGTGACGTACACC	56.0	
OPG	CAAAGTAAACGCAGAGAGTGTAGA	59.6	AF_134187
	GAAGGTGAGGTTAGCATGTCC	59.9	
IL-1β	CTTCTTCGACACATGGGATAAC	54.2	NM_000576
	TTTGGGATCTACACTCTCCAGC	56.4	
IL-4	GTTGACCGTAACAGACATCTTTG	60.2	NM_172348
	CGAGCCGTTTCAGGAATC	59.9	
IL-6	CTAGAGTACCTCCAGAACAGATTTGA	60.2	NM_000600
	TCAGCAGGCTGGCATTT	60.9	
MMP-2	CGACCGCGACAAGAAGTA	60.2	NM_004530
	GCACACCACATCTTTCCGTCA	60.4	
MMP-9	CAGTTTCCATTCATCTTCCAAGG	59.9	NM_004994
	CCCATCACCGTCGAGTC	60.2	

ALP, alkaline phosphatase; BSP, bone sialoprotein; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (reference gene); IL, interleukin; MMP, matrix metalloproteinase; OCN, osteocalcin; OPG, osteoprotegerin; OPN, osteopontin; RANKL, nuclear factor-κB ligand.

Statistical analysis

The data were averaged and tested by an intergroup analysis using the Student's *t*-test ($\alpha = 0.05$) (e.g. control vs. test). The RANKL : osteoprotegerin proportion was tested using the Mann–Whitney test ($\alpha = 0.05$).

Results

Clinical observations

During the healing period, no abscess formation or suppuration was observed. Although primary soft tissue closure was obtained in all sites, gingival recession (2 mm), with exposure of the coronal part of the membrane, was observed in only one site of the test group. Postsurgical care was provided by topical application of 1% chlorhexidine gel; this site showed no further complications and was included in the data analysis. At the end of the experimental period (e.g. 21 d after the surgical procedure) soft tissues had totally healed and exhibited no clinical signs of inflammation.

Gene expression analysis

In order to determine whether the presence of the membrane would modulate gene expression *in vivo*, the regenerating tissue formed in the defects in the control and test groups was assessed and compared for the expression of the following genes: alkaline phosphatase, RANKL, osteoprotegerin, osteopontin, osteocalcin, bone

sialoprotein, basic fibroblast growth factor, interleukin-1, interleukin-4, interleukin-6, MMP-2 and MMP-9. Data analysis demonstrated that, except for interleukin-4 ($p > 0.05$), all genes were differentially expressed in the membrane-protected sites and nonprotected sites, suggesting that the mRNA levels for all the factors were significantly affected by the presence of the membrane. Alkaline phosphatase, RANKL, osteoprotegerin, osteopontin, bone sialoprotein, basic fibroblast growth factor, interleukin-1, interleukin-6, MMP-2 and MMP-9 levels were higher in the sites where the guided tissue regeneration principle was applied, compared with the sites where only open flap debridement was used ($p < 0.05$). By contrast, the mRNA levels for osteocalcin were lower in the test group compared with the control group ($p < 0.05$). Finally, an increased osteoprotegerin : RANKL ratio was found in nonprotected sites vs. membrane-protected sites ($p < 0.05$). Figures 1–3 illustrate gene expression data.

Discussion

The regenerative techniques currently available are unpredictable, reflecting the complexity of periodontal wound healing, which involves the interaction of distinct tissues and diversity in the activities of cells, coupled with the marked variation in the way that individuals respond to injury. Critical to improving the predictability of regenerative therapies is the targeting of studies towards enhancing our understanding of the cellular and molecular events required to restore the periodontal tissues. Previous studies have demonstrated, by immunohistochemical techniques, that extracellular matrix molecules associated with wound healing and/or remodelling are more strongly expressed in regenerated tissues than in intact tissues (3,5,9,10,14), but failed to reveal differences in terms of staining intensity or distribution pattern of the investigated matrix molecules between guided tissue regeneration, enamel matrix proteins, the combination of both, or access flap surgery (5). Additional studies assessed gene expression in primary and cloned cultures of human periodontal cells from different sources (regenerating tissue, periodontal ligament and gingiva) under guided tissue regeneration conditions and identified markers that correlate with the known ability of periodontal ligament and regenerating tissue-derived fibroblasts to regenerate the mineralized tissues of the periodontium (10,15). However, to date it is not known whether genes related to periodontal regeneration are differently expressed in nonprotected surgical periodontal healing sites vs. membrane barrier-led healing sites. Although limited to one time point after surgery for root access and membrane placement (21 d), the results of the present study demonstrated, in humans, that the presence of a physical barrier to prevent the invasion of epithelial cells in the defect area may modulate gene expression in the regenerating tissue formed. Many of the genes assessed have been shown to be involved in connective tissue wound healing and mineralized tissue formation, thus confirming the results of previous reports (16).

In the periodontal tissues, both repair and regeneration are regulated by the local production of growth factors. Growth factors are natural biological molecules that mediate and regulate key cellular events during tissue repair, including cell proliferation, chemotaxis, differentiation and matrix synthesis, by binding to specific cell-surface receptors (17,18). Basic fibroblast growth factor is a member of the fibroblast growth factor family, is a potent mitogen and is chemoattractive for endothelial cells and a variety of mesenchymal cells, including fibroblasts and osteoblasts, and therefore is an important factor in the early steps of the healing process. *In vitro* studies have shown that basic fibroblast growth factor stimulates mitogenic activity and chemotaxis in periodontal ligament cells (19). Additionally, *in vivo* studies have suggested a beneficial effect of basic fibroblast growth factor on matrix formation during the bone healing process (20,21). In the present study, mRNA levels for basic fibroblast growth factor were upregulated at the test sites when compared with nonmembrane-protected sites, suggesting that basic fibroblast growth factor may play an important role during the early periodontal healing phase in membrane-protected defects.

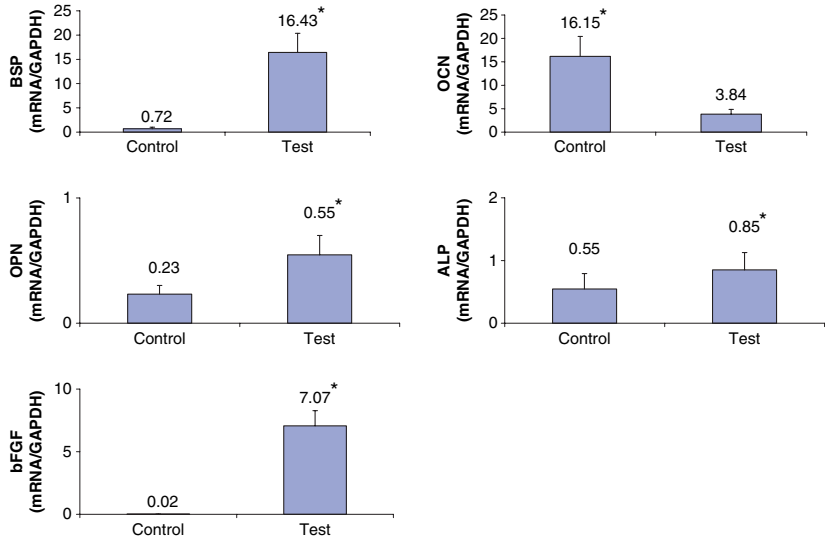

Fig. 1. Bar charts showing the mean values and standard deviation of the mRNA levels of the following mineral-associated factors and growth factors: bone sialoprotein (BSP), osteocalcin (OCN), osteopontin (OPN), basic fibroblast growth factor (bFGF) and alkaline phosphatase (ALP) for the control and test sites. *Indicates that there is a statistically significant difference between groups ($p < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

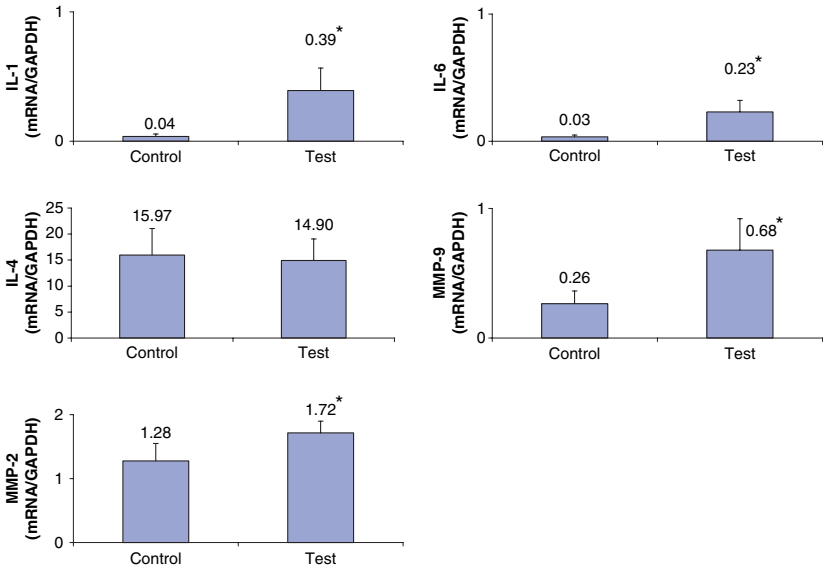

Fig. 2. Bar charts showing the mean values and standard deviation for the mRNA levels of genes related to the inflammatory process and extracellular matrix degradation, including: interleukin-1, interleukin-4, interleukin-6, matrix metalloproteinase-2 and matrix metalloproteinase-9 for the control and test sites. *Indicates that there is a statistically significant difference between groups ($p < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MMP, matrix metalloproteinase.

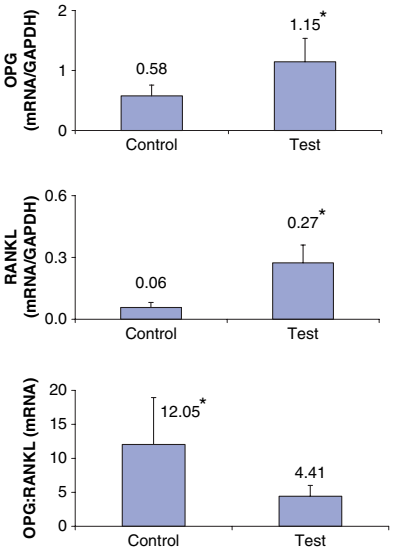

Fig. 3. Bar charts showing the mean values and standard deviation for the mRNA levels of molecular regulators of bone metabolism: osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL) and the OPG : RANKL ratio for the control and test sites. *Indicates that there is a statistically significantal difference between the groups ($p < 0.05$).

In the present study, the mRNA levels for bone/cementum-associated factors, including alkaline phosphatase, bone sialoprotein, osteocalcin and osteopontin, were also assessed. Alkaline phosphatase, bone sialoprotein and osteopontin mRNA levels were found to be higher in the sites where guided tissue regeneration was applied, whereas osteocalcin mRNA levels were lower. The activity of alkaline phosphatase is considered to be an important indicator of bone formation and a phenotypic marker of osteoblast cells (22). Previous studies have already demonstrated that alkaline phosphatase activity is upregulated in regenerated human periodontal cells obtained from retrieved e-PTFE membranes of periodontal disease patients and from the regenerated tissue underlying the membrane (23). Osteopontin is a differentiation marker for the osteoblastic and cementoblastic cell phenotype (24); and bone sialoprotein and osteocalcin are highly specific to mineralized tissues, including bone and developing and mature cementum (25,26). Evidence to date suggests that bone sialoprotein is a nucleator of hydroxyapatite crystal formation, while osteocalcin appears to play a role in the early phases of mineralization and in the regulation of crystal growth (27).

In the present investigation, the higher level of expression of alkaline phosphatase, osteopontin and bone sialoprotein in the newly formed tissue in the membrane-protected intrabony defects suggests that there is a marked intrinsic potential for hard tissue regeneration in such an area, and therefore the findings of the present study suggest that periodontal regeneration may additionally be modulated by membrane through its effect on the expression of these mineral-associated genes.

Molecular regulators of bone metabolism, including osteoprotegrin and RANKL, were also assessed in this study. Interestingly, both osteoprotegerin (anti-resorptive factor) and RANKL (pro-resorptive factor) mRNA levels were higher in the test group than in the control group. More importantly, the RANKL : osteoprotegerin ratio was significantly increased in the control group, indicating a higher catabolic activity in the membrane-protected sites. Recent studies have demonstrated that the RANKL : osteoprotegerin system may take part in periodontal regeneration (28,29); however, to the authors' knowledge, this is the first study to show a possible modulation of the RANKL : osteoprotegerin mRNA levels at periodontal defects by a physical barrier. These findings support a role for membrane stimulation of mineralized tissue formation that is consistent with periodontal regeneration by modulating regulatory molecules critical to bone metabolism at the mRNA level.

Finally, genes related to the inflammatory process and matrix degradation were also investigated, such as interleukin-1, interleukin-4, interleukin-6, MMP-2 and MMP-9. Both interleukin-1 and interleukin-6 are reported to induce osteoclastogenesis by modulating RANKL expression (30) and they also increase MMP) production, which plays a critical role in depredating and remodeling connective tissue (31). In the present study, higher levels of mRNA were found in the test group for all of the investigated inflammatory and matrix deposition-related genes, except for interleukin-4,

which was expressed at similar levels in both groups.

In conclusion, within the limits of the present study, the findings demonstrated that a set of functionally relevant genes may be differentially regulated in membrane barrier-led periodontal healing in favor of periodontal regeneration. Future studies, however, should be considered in order to investigate in greater detail the role of barrier membranes in periodontal regeneration and to understand the mechanisms involved, in a time-sequence manner. This knowledge may provide information on ways in which the periodontal wound-healing process can be manipulated to achieve predictability in periodontal regeneration.

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