



Morphological and phenotypic differences in fibroblasts obtained from gingival overgrowth secondary to phenytoin: A pilot study

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ABSTRACT

Introduction: Gingival fibroblasts seem to be key cells in the pathogenesis of gingival overgrowth secondary to phenytoin intake. These cells not only participate in the extracellular matrix protein synthesis, but also regulate protein degradation through production of collagenolytic enzymes and phagocytosis. Some growth factors and cytokines such as IL-1 α , TGF- β and TNF- α participate in the homeostasis of extracellular matrix. **Objective:** The aim of this study was to evaluate the presence of IL-1 α , TGF- β and TNF- α receptors on human gingival fibroblasts from a healthy donor, a patient with gingival overgrowth consuming phenytoin (responder) and a donor consuming phenytoin without gingival overgrowth (non-responder). **Methods and results:** Primary cell cultures of gingival fibroblasts from each donor were characterized by flow cytometry demonstrating vimentin and CD14 positive cells. Using size detector, gingival fibroblasts from the responder donor were larger than fibroblasts from non-responder and healthy donor. A low but positive expression of IL-1R and TGF- β R was detected in cells from the responder donor. TNF- α R expression was detected in all three types of cultured cells but it was higher in the responder donor. **Conclusions:** These results suggest differences in the morphologic and phenotypic characteristics between gingival fibroblasts from the donors studied.

Key words: Gingival fibroblast, cytokine, phenytoin.

Palabras clave: Fibroblasto gingival, citocinas, fenitoína.

RESUMEN

Introducción: Los fibroblastos gingivales son considerados como células claves en la patogénesis del agrandamiento gingival secundario al uso de medicamentos como la fenitoína. No solamente participan en la síntesis de proteínas de matriz extracelular, sino también en su degradación mediante la producción de enzimas colagenolíticas y fagocitosis. Algunas citocinas como la IL-1 α , TGF- β y TNF- α participan en la homeostasis de la matriz extracelular. **Objetivo:** El objetivo de este estudio fue evaluar la presencia de receptores para IL-1 α , TGF- β y TNF- α en fibroblastos gingivales humanos de un donante sano, un paciente con agrandamiento gingival consumidor de fenitoína (respondedor) y un donante consumidor de fenitoína sin agrandamiento gingival (no respondedor). **Metodología y resultados:** Cultivos primarios de fibroblastos gingivales obtenidos de cada paciente fueron caracterizados por citometría de flujo demostrando células positivas para vimentina y CD14. Usando el detector de tamaño por citometría, los fibroblastos gingivales del paciente respondedor mostraron mayor tamaño que las células de los otros sujetos. Se detectó una expresión positiva pero baja para IL-1R y TGF- β R en células del donante respondedor. La expresión del TNF- α R fue detectada en los tres tipos de poblaciones celulares, pero ésta fue mayor en fibroblastos del donante respondedor. **Conclusión:** Estos resultados sugieren diferencias morfológicas y fenotípicas entre las poblaciones de fibroblastos gingivales estudiados.

INTRODUCTION

One of the most common side effects secondary to phenytoin consumption in patients with therapy for epileptic syndrome is gingival overgrowth.¹ Nearly 50% of patients on phenytoin develop altered enlargement of the gingival tissue. Previous studies have proposed several mechanisms participating in this gingival overgrowth, such as: a) direct association between serum and salivary levels of phenytoin;¹ b) presence of dental plaque; related more with the severity of the disease;² c) low levels of salivary IgA in phenytoin consumers;³ d) increase of fibroblast proliferation and protein synthesis mediated

by steroid hormones;⁴ or e) augmentation of local levels of IL-1 and proliferation of Langerhans cells in the gingival epithelia.⁵

Abbreviation:
ECM: extracellular matrix

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Since all patients consuming phenytoin do not develop gingival overgrowth other factors such as environmental stimuli and genetic predisposition must be involved. The term "responder" is applied to patients under phenytoin therapy that develop gingival overgrowth and "non responders" to those under phenytoin therapy who do not develop this tissue enlargement. Evidence suggested that a functional heterogeneity of the human gingival fibroblast exist in both groups of patients and this has been further supported by their response to different stimuli.⁶ One of the more important features of gingival overgrowth, even more significant than the increase in the number of cells, it is the augmentation of "extracellular matrix (ECM) proteins" such as collagen.^{6,7} This latter histological evidence favors the hypothesis that phenytoin possibly breaks the homeostasis in the ECM, increasing synthesis of connective proteins or inhibiting their degradation. Previous data support this hypothesis, showing an upregulation in the synthesis of type I and II collagen and glycosaminoglycans during gingival overgrowth. Furthermore, impaired secretion of ECM degrading enzymes, such as Matrix Metalloproteinases (MMPs) or their Tissue Inhibitor of Metalloproteinase (TIMPs) has also been documented.⁷

Different cell populations found in the ECM produce growth factors such as PDGF, EGF, bFGF^{8,9} and "fibrogenic" cytokines including TGF- β , TNF- α and IL-1 α which seem to be implicated in the development of tissue overgrowth. TGF β promotes ECM protein synthesis, including type I, III, IV, VI and X collagen. Recently, it was shown that phenytoin in association with TNF- α induces IL-1 α secretion by gingival fibroblasts. IL-1 α in conjunction with TNF- α induces enzymatic synthesis of collagenase, which is necessary for ECM degradation, activity that appears to be mediated by PGE₂.^{10,11}

Local increase of fibrogenic cytokines could be implicated in the pathogenesis of gingival overgrowth secondary to phenytoin. Differential expression in cytokine receptors on human gingival fibroblasts could be useful in the identification of a possible "fibrotic phenotype" associated with connective tissue disorders.

In this study we analyzed the morphological and phenotypic characteristic of human gingival overgrowth *in vitro* from one healthy donor, one subject consuming phenytoin without gingival overgrowth (non responder) and one patient with gingival overgrowth secondary to phenytoin treatment (responder). Differences in the cell size and cytokine receptor expression in fibroblasts were found comparing donors primary cultures.

MATERIAL AND METHODS

Donors and gingival sampling

Gingival biopsies were obtained from three individuals who had previously signed an informed consent form in agreement with the ethics committee of the Universidad Javeriana. Selection criteria included absence of clinically detectable periodontal inflammation, non-smokers and no signs and symptoms of systemic disease. The subjects were classified as: one healthy donor (male 30 years), one non-responder (male 40 years) and one responder (male 43 years). Twenty-four hours before the surgical procedure, a prophylaxis was done with 0.2% chlorhexidine. The biopsies were taken during esthetic gingivectomy from normal and non-responder donor and gingivoplasty from responder donor. The tissues were rinsed three times in 0.85% saline solution and transported in Minimal Essential Medium (MEM) (Sigma, St. Louis, MO), with penicillin 100 U/mL and streptomycin 100 μ g/mL (Sigma).

Human gingival fibroblasts cultures

Samples were cut in small pieces and explants were placed in a 25 cc culture flask (Falcon) and incubated for 10 minutes at room temperature. Then, samples were added to MEM containing 10% Fetal Calf Serum (FCS) (GIBCO BRL, Life Technologies, Grand Island NY), and antibiotics (complete medium). Cells were maintained at 37°C and 5% CO₂. Cells were passed every week by removal with 0.25% trypsin and 1mM EDTA. All experiments were carried out with cells between the fourth and eight passages. For analysis of surface markers and cytokine receptors, cells were detached using 20 mM EDTA. Viability of the cells was evaluated by trypan blue exclusion.

Characterization of cell cultures

After the fourth passage, human gingival fibroblasts were stained for vimentin and CD14 expression. For surface staining, 1 x 10⁵ cells were washed with 0.01M PBS, 1% FCS, 0.1% NaNO₃. The pellet was allowed to react with 5 μ L of antihuman CD14 PE (Becton Dickinson, San Jose, CA) for 30 minutes at 4 °C. Isotype PE matched antibody was used as control. Cells were washed and fixed with 0.01M PBS pH 7.4 and 1% paraformaldehyde. For cytoplasm staining, a total of 1 x 10⁵ cells were fixed and after washing, cells were permeabilized with 0.01M PBS and 1% saponin. Following permeabilization, 10 μ L of anti-vimentin (Sigma) antibody was added and cells were incu-

bated in the dark for 30 minutes at 4 °C. After washing, 10 μ L of a 1/40 dilution of FITC labeled anti-mouse IgM (Sigma) antibody was added and cells incubated as previously described. As a negative control for vimentin, permeabilized cells were incubated with matched isotype control. Cells were acquired with a FACScalibur (Becton Dickinson, San Jose, CA) and analyzed using Cell Quest software.

Analysis of cytokine receptors expression on fibroblast

For detection of each cytokine receptor, 2×10^5 cells were stained for human IL-1 α , TGF β and TNF- α receptors with FLUOROKINE™ (R & D Systems, McKinley Place, MN) according to the manufacturer instruction with some minor changes. Briefly, cells were incubated with 10 μ L of biotinylated cytokine (IL-1 α , TGF β 1 and TNF- α) for 1 hour at 4 °C and washed with buffer (provided with the kit). Labeled cells were incubated with 10 μ L of avidin-FITC 30 minutes at 4°C in the dark. At least 10,000 cells were analyzed by flow cytometry and each experiment by was run in duplicate.

RESULTS

After 5 days of culture all three donors derived tissue epithelial-like cells emerged from explants and were replaced at day 7 by enlarged and spindle shaped cells resembling fibroblasts. After the third passage (3 weeks) cells formed a monolayer typical of fibroblasts (*Figure 1A*). Characteristic fibroblast morphology was observed by light microscopy as shown in *figure 1B*. Cells in fourth passage were used to establish the size, granularity and expression of markers by flow cytometry.

By flow cytometry analysis, cells were typically located in the center of forward scatter (FSC) versus side scatter (SSC) plot. Interestingly, cells from responder donor had higher FSC mean intensity. This feature was also detected by light microscopy. Region 1 or R1 was the area where most live cells were located and excluded dead cells (*Figure 2A*). Therefore, R1 was selected for further analysis. Expression of the intracellular marker vimentin, exclusive to cells of mesenchymal origin,^{12,13} was positive in the cultured cells (*Figure 2B*). No differences were observed in vimentin expression in the three different fibroblasts cultures as judge by mean fluorescence intensity (MFI). Expression of surface CD14 was also positive on cultured cells (*Figure 2c*). There were no differences in the expression of CD14 between gingival fibroblasts from all three donors, but expression was remarkably lesser than that found in human monocytes (data not shown).

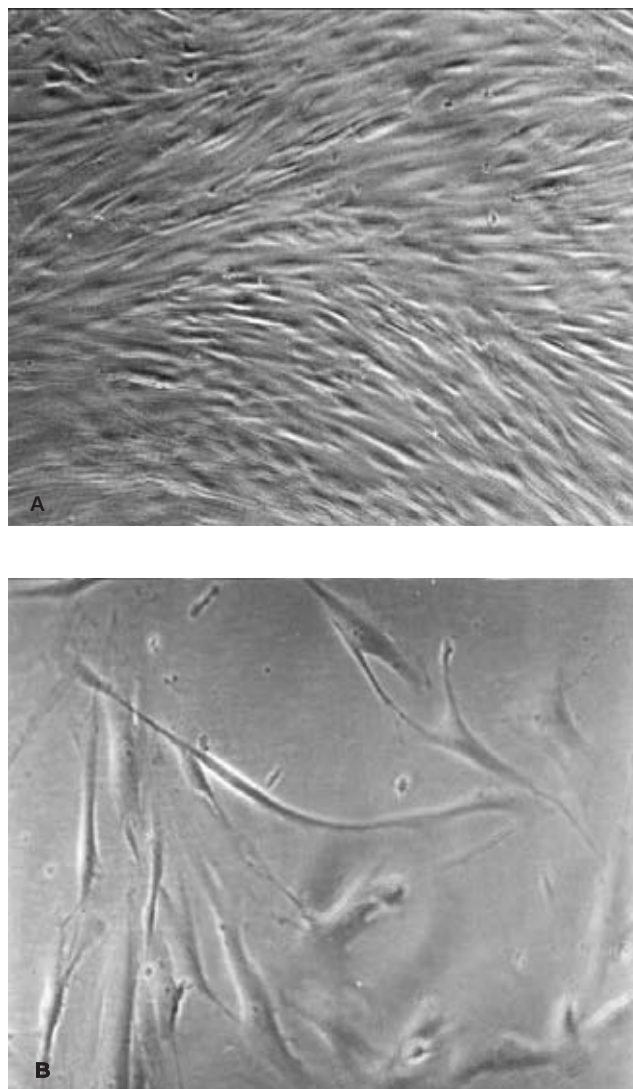


Figure 1. Cultures of human gingival fibroblasts from healthy donor. **A.** Monolayer of enlarged cells resembling fibroblasts (10x). **B.** Typical morphology of fibroblast (20x).

Comparison on the MFI of size detection (FSC) showed a statistically significant difference between the gingival fibroblasts from all sources. Thus, cells derived from explants of responder donor were larger (mean $502,7 \pm 36,5$; number of individual cultures analyzed $n = 41$) compared to non-responder (mean $465,7 \pm 39,7$; $n = 45$) with a $P = 0.001$; or healthy donor (mean $450,7 \pm 33,4$, $n = 50$) with a $P = 0.001$ and between non responder and healthy donor $P = 0.049$, by T-student test. These results corroborate the findings observed by light microscopy.

Before cytokine receptor staining in gingival fibroblasts, the system was tested with resting and mito-

gen (PMA) activated human monocytes (CD14⁺ cells). IL-1R and TNF- α R were detected in resting cells and TGF β R in PMA activated monocytes (data not shown). Analysis of cytokine receptors were initially assessed comparing control condition (cells + avidin/FITC alone) and experimental conditions (cells + cytokine/biotin and avidin/FITC). No expression of IL-1R and TGF β was observed in fibroblasts from healthy donor (Figure 3). Expression of IL-1R and TGF β R was slightly positive in cell from non-responder donor; both receptors were higher expressed in cell from the responder donor measured as MFI. TNF- α R was the most positive receptor detected in all three gingival fibroblasts populations and expression was higher in responder donor (Figure 3). Similar MFI values were found for TNF- α R expression in healthy and non-responder donor.

DISCUSSION

In this pilot study, human gingival fibroblasts were obtained and characterized from three sources: a) healthy donor, b) a patient under phenytoin therapy without gingival overgrowth or non responder, and c) a patient under phenytoin therapy with gingival overgrowth or responder donor. Cells in culture were assessed for the expression of the cytokines receptors for IL-1, TGF β and TNF- α . Previous studies demonstrated differences between fibroblast cultures among individuals.¹⁴ For that reason only cultures from each type of donor were tested here.

When size and internal complexity of human gingival fibroblasts were analyzed by flow cytometry using forward and side scatter detection, the cells had a medium size and low granularity. This is in contrast with fibroblast from the periodontal ligament which are larger and more granular.¹⁵ Based on light microscopy, cells in responder donor were larger compared with cultures from healthy and non-responder donors. This qualitative feature was corroborated by analyzing the cells between the fifth and sixth passage by flow cytometry. This finding could indicate a higher capacity of the cells from the responder donor to produce and secrete ECM proteins and soluble factors. Indeed, morphologic studies have demonstrated collagen accumulation in drug induced gingival overgrowth. In contrast, phenytoin reduces collagen synthesis in gingival fibroblasts from healthy donors.¹⁶

Expression of intracellular vimentin and surface CD14 was positive as previously reported.¹³ There were no differences in the expression of these markers between cultured gingival fibroblasts from all three different patients. Expression of CD14 in gingival fibro-

blasts has been assessed using immunocytochemistry and western blot.^{17,18} In this report, the previous observations are supported by flow cytometry. This finding will allow the development of functional studies of gingival fibroblast responses to bacterial products such as LPS from oral pathogens such as *P. gingivalis*, *F. nucleatum* and *T. forsythia* as well as its interaction with CD14 or Toll like receptors 2 and 4 (TLR-4). These cellular activation pathways could not only be implicated in the pathogenesis of oral chronic inflammatory disorders (e.g. Periodontitis), but also associated to impaired MEC metabolism during gingival overgrowth secondary to phenytoin.¹⁷

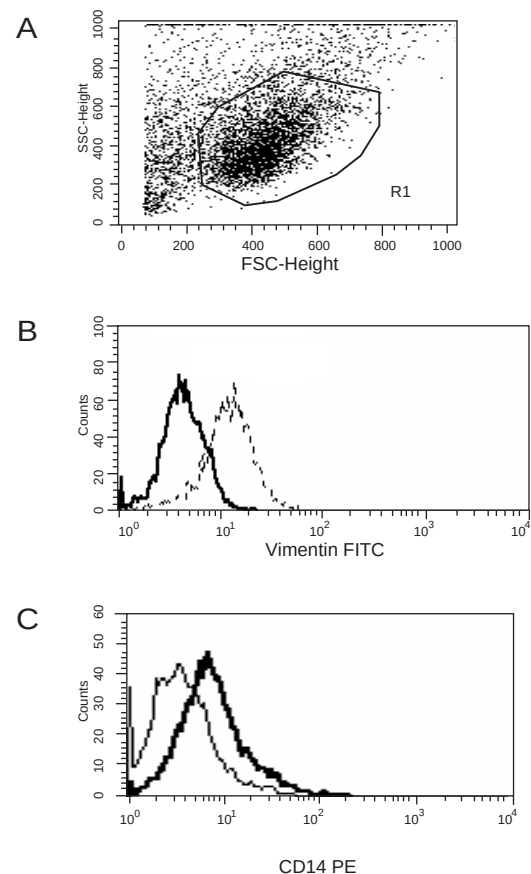


Figure 2 A. Dot plot of flow cytometric analysis showing size (FSC) by internal complexity (SSC). Region 1 (R1) was selected for further analysis. B. Histogram obtained by flow cytometry showing intracellular expression of vimentin. Continuous line indicates the control antibody and the dotted line indicates the staining with anti-vimentin antibody. C. Histogram obtained by flow cytometry showing surface expression of CD14. Thin line indicates isotype matched antibody and bold line indicates anti-CD14 antibody labeling.

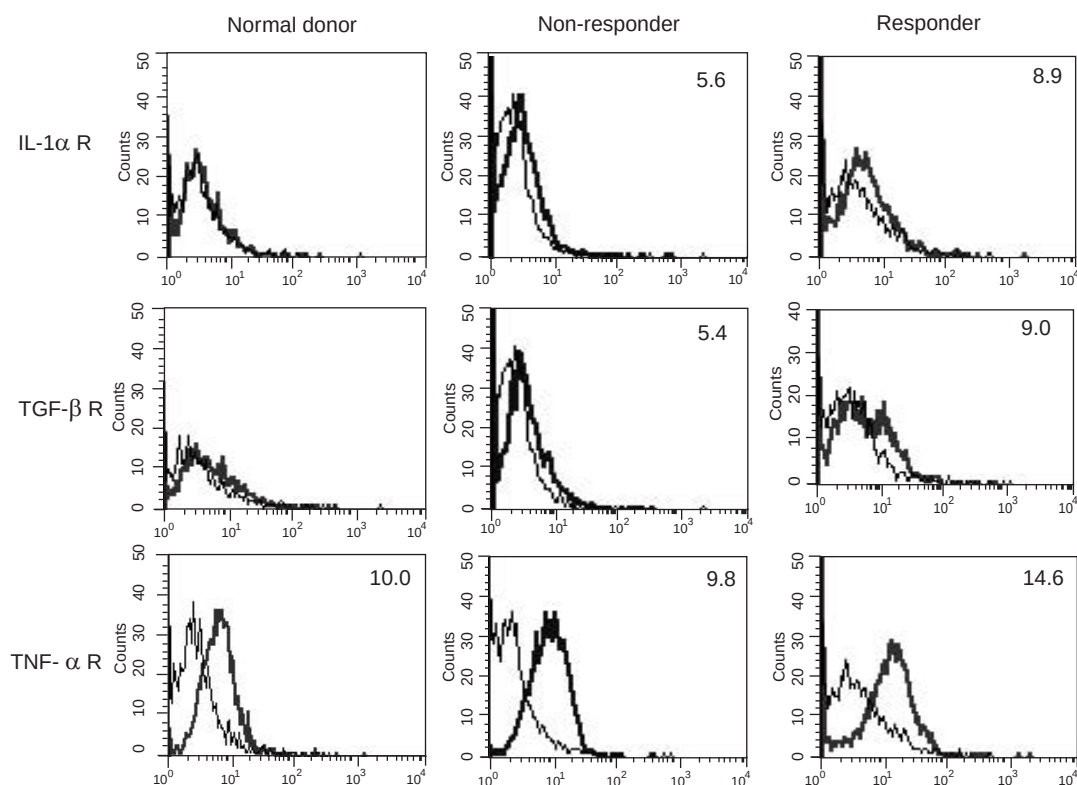


Figure 3. Expression of cytokine receptors in gingival fibroblasts, one experiment out of three done is shown. Histogram obtained by flow cytometric detection of FITC fluorescence. Files indicate labeling with the respective cytokine i.e., IL-1 α , TGF β or TNF- α . Columns indicate the source of fibroblasts. For each histogram thin line indicates control cells and bold line indicates labeling with fluorescent cytokines. Values of Mean Fluorescence Intensity (MFI) for each respective cytokine receptor are shown.

In vitro effects of cytokines (IL1- α , TGF β and TNF- α) on gingival fibroblasts have shown impaired ECM homeostasis, implicating changes in cell proliferation, protein synthesis and protein degradation.^{11,19-22} Thus, changes in the expression of cytokine receptors at the fibroblast level could be important in the pathogenesis of overgrowth of the gingival tissue.

Fibroblasts from healthy donor did not express TGF β R; however TGF β R was identified at low level in cell from non-responder and highly expressed in cells from responder donor. Previous work demonstrated an immunochemical increase of TGF β R type I and II in gingival tissue of overgrowth secondary to cyclosporin compared with healthy gingival tissue.²² TGF β 1 does not induce proliferation of gingival fibroblast but promotes synthesis and secretion of ECM proteins.^{11,23} Interestingly, the interaction of TGF β 1 with cyclosporine reduces the production of MMP-1 and MMP-2.²⁴

In gingival fibroblasts, IL-1R has a similar pattern of expression as TGF β R. IL-1R could increase binding of IL-1 produced by local immune cells (paracrine) and

gingival fibroblast (autocrine) that regulate ECM homeostasis. High doses of IL-1 β (300 to 500 pg/mL) reduce collagen production^{25,26} but also increase the synthesis and secretion of ECM degrading enzymes such as MMP-1, MMP-2 and MMP-3.^{21,25,27} Interestingly, as a control mechanism, some of these MMPs are able to cleave and inactivate IL-1 β .²³ However, the interactions between IL-1 β with drugs that induce gingival overgrowth produce different outcome in normal fibroblasts. Thus, phenytoin enhances the inhibitory effect of IL-1 β on collagen production.²⁸ By contrast, interaction IL-1 β with nifedipine increases collagen synthesis.²⁶

Expression of TNF- α R was positive in all three types of human gingival fibroblasts as reported here. However, TNF- α R expression was higher in cells from responder donor. An increase in this receptor expression in fibroblasts from gingival overgrowth secondary to phenytoin may be associated with impaired collagen degradation via extracellular mechanisms such as proteases or intracellular mechanisms such as phagocytosis, since low concentrations of TNF- α (10 ng/

mL) in gingival fibroblasts inhibited the phagocytic degradation, blocking collagen receptors ($\alpha 2 \beta 1$ integrins), and increasing the synthesis of MMPs. High TNF- α concentration (500 ng/mL) reduced collagen synthesis, but its synthesis is augmented in the presence of nifedipine.²⁹

Differential expression of cytokine receptors in fibroblasts from the "responder" donor regards to fibroblasts from "non-responder" donor may be potentially involved in the pathogenesis of this tissue enlargement associated with phenytoin. Thus, up-regulation of TGF β R could be likely favoring collagen production as well as reducing enzymatic ECM-degradation. In addition, increased TNF- α R expression could be accounting for diminished ECM degradation via phagocytosis. Finally, IL-1 may act as a compensation mechanism reducing protein synthesis and increasing MMPs.

In the present pilot study expression of the IL-1R, TGF β R and TNF- α R in human gingival fibroblasts was assessed for the first time. Secretion of particular cytokines accompanied by differential expression of cytokine receptors have been shown to be critical mechanisms controlling protein synthesis and/or degradation of the ECM. Therefore, an impaired balance of it may play a role in the pathogenesis of the gingival overgrowth secondary to intake of phenytoin and possibly other drugs.

ACKNOWLEDGEMENTS

The authors thank Dr. Nelson Contreras for his continued support, Drs. Luz K. Sánchez, Andres Pinto, and Camilo Durán for the reading of the manuscript, Bogotá's Liga Central Contra la Epilepsia and to the patients who participated in this study. Special thanks to K.C Dowdell and L. Hamo, School of Medicine, USC, Los Angeles, for correcting the manuscript. This work was founded by the Dental Research Center, School of Dentistry, Pontificia Universidad Javeriana.

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