Effect of Local Nifedipine Administration on Rat Gingiva

Davide Bencivenni[1], Mirdza E. Neiders[2], Sebastiano Andreana[3], Michelle L. Moffitt[4] and Robert E. Cohen[4]

[1]School of Dental Medicine, University of Modena and Reggio Emilia, Modena, Italy; Departments of [2]Oral Diagnostic Sciences, [3]Restorative Dentistry, and [4]Periodontics and Endodontics, University at Buffalo, The State University of New York, School of Dental Medicine, Buffalo, NY

Abstract

Background: Nifedipine, a calcium channel-blocking agent, has been associated with gingival enlargement in humans. This enlargement has also been successfully established in animal models. Previous investigators have administered nifedipine through a systemic route, most commonly by oral intake. The aim of the present study was to measure the effects of nifedipine administered directly into rat gingival interproximal papillae. **Methods:** Twenty-four adult female rats were assigned to three groups. Each animal received a series of three injections, one week apart; each injection was placed directly into the interdental papilla of the maxillary and mandibular central incisors. Group 1 (control) received only saline. Group 2 received a low (10 µg/ml) concentration of nifedipine, while Group 3 received a higher concentration (500 µg/ml). One week after the last series of injections, gingival specimens were harvested from the injection site and prepared for histological and immunocytochemical analyses. **Results:** Specimens from Group 3 displayed a significantly greater number of ED2-positive cells compared to the other two groups. Specimens from Group 2 showed a significantly higher mean count of positive cells compared to Group 1. Collectively, our data suggest that repeated local injections of 10 µg/ml and 500 µg/ml nifedipine each elicit an inflammatory response in the gingival connective tissue. **Conclusions:** Immunocytochemical analysis revealed dose-dependent increases of resident tissue macrophages in rats receiving nifedipine ($p < 0.005$). An increased inflammatory infiltrate also was observed via routine histology. Gross macroscopic changes consistent with gingival enlargement were not observed.

Key words: Nifedipine, calcium channel blocking agents, immunosuppressant, anticonvulsants, gingival enlargement

Introduction

Drug-induced gingival enlargement is typically characterized by a thickening of the spinous layer of the gingival epithelium and increase in collagen deposition (Bulut *et al.*, 2006). Although the mechanism of drug-related gingival enlargement has not been completely elucidated, it involves an interaction between a drug and resident gingival fibroblasts, resulting in a lower rate of collagen phagocytosis (Shimizu *et al.*, 2002). Additional factors also might contribute to gingival enlargement, including age, sex, plaque, genetic predisposition and local tissue characteristics (Guncu *et al.*, 2007).

Correspondence to: Mirdza E. Neiders, Department of Oral Diagnostic Sciences, University at Buffalo, The State University of New York, School of Dental Medicine, 250 Squire Hall, Buffalo NY 14214. E-mail: neiders@buffalo.edu.

Drug-induced gingival enlargement was first associated with phenytoin in 1939, but since has been associated with three different therapeutic classes of drugs: calcium channel blockers, immunosuppressants, and anticonvulsants (Lin *et al.*, 2007). Although those classes are unrelated to one another, they are all known to affect intracellular calcium levels by inhibiting its entry into the cell (Gelfand *et al.*, 1986; Messing *et al.*, 1985). The decreased intracellular Ca^{2+} level seems to play a crucial role in the pathogenesis of gingival overgrowth. Intracellular calcium is required by fibroblasts for the synthesis and secretion of enzymes involved in collagen degradation, such as matrix metalloproteinases (MMPs). The upregulation of MMP gene expression is specifically triggered by protein kinase C, via induction of the binding of the

transcription factor AP-1 to a specific promoter sequence of the MMP gene (Birkedal-Hansen, 1993). Activation of protein kinase C is a calcium-dependent process (Hardie *et al.*, 1990; Shimizu *et al.*, 2002). This pathway may explain, at the transcriptional level, how each of the drugs associated with gingival overgrowth alters the sequence of events that lead to MMP expression and release from gingival fibroblasts. The diminished collagen breakdown results in a shift of the homeostatic balance and a net accumulation of extracellular matrix becomes clinically evident as gingival overgrowth. This hypothesis has been confirmed by reduced MMP-1 staining in drug-induced gingival overgrowth tissue samples (Walters, 1993). Fujii (1990) also showed the ability of nifedipine to inhibit cellular calcium uptake in gingival fibroblasts.

Numerous studies have demonstrated that clinical manifestations of drug-induced gingival overgrowth are similar in animals and humans (Nishikawa *et al.*, 1996; Nishikawa *et al.*, 1991). The gingival papilla and the gingival margin typically become enlarged, and are characterized by firm, red, nodular tissue. Sibling animals offer the advantage of minimizing the interpatient variability due to genetics or other individual patient characteristics. Availability, ease of handling and cost make rats one of the most suitable animal models. The rat bears close resemblance to humans with respect to periodontal anatomy, development and composition of oral plaque, and histopathology of periodontal disease (Nishikawa *et al.*, 1996; Nishikawa *et al.*, 1991; Shaker *et al.*, 2011).

Experimental induction of drug-related gingival overgrowth in rats has been obtained by many investigators (Kataoka *et al.*, 2001; Nishikawa *et al.*, 1996; Nishikawa *et al.*, 1991). Nishikawa *et al.* (1996) administered nifedipine (250 μg/g diet) to rats and observed macroscopic enlargement as early as 20 days after oral ingestion. Longer treatment periods of up to 70 days did not result in increased severity of the overgrowth. It also was observed that when the drug was removed from the animals' diet, the gingival macroscopic morphology returned to control levels within 30 days (Nishikawa *et al.*, 1991). The authors concluded that several clinical features were common to gingival overgrowth induced by calcium channel blockers, phenytoin or cyclosporin A in rats: 1) a more conspicuous enlargement of the buccal rather than the lingual gingival; 2) less severe enlargement of the maxilla than the mandible; 3) accumulation of dental plaque influences the severity, but is not essential for the onset of overgrowth; and 4) more severe overgrowth is observed in young rats. It was determined that severity is dependent upon drug blood levels, as well as the duration of drug administration, with maximum overgrowth developing between 30 and 40 days (Nishikawa *et al.*, 1991). Since those same factors have been hypothesized as being important in drug-induced gingival overgrowth in humans, the rat model may be a valuable model for investigating drug-induced gingival enlargement.

Histological analysis of nifedipine-induced, enlarged gingival tissue reveals that both the epithelium and the connective tissues are affected. The lamina propria typically displays an increased vascularity and a chronic inflammatory cell infiltrate. Bundles of immature collagen fibres are densely packed or loosely textured. The epithelium is thickened and acantotic (Nishikawa *et al.*, 1996; Nishikawa *et al.*, 1991; Shaker *et al.*, 2011). Histomorphometric analysis also indicates that responder rats have a 2.5-fold increase in mean cross sectional gingival area (Nishikawa *et al.*, 1991).

Previous human or animal studies generally have been performed to investigate the incidence of and the mechanisms of systemically administered drug-induced gingival overgrowth. To date, there are no studies that attempt to exploit this pharmacologically induced tissue change in order to purposely regenerate gingival tissue. To the best of our knowledge, there are no animal studies where drugs have been delivered locally or topically to specific sites. Consequently, the aim of this study was to measure the effects of local nifedipine delivery on gingival tissues using macroscopic observation, as well as through quantitative immunocytochemical analysis of a macrophage subset that may serve as a marker of fibroblast activation.

Materials and methods

This study was reviewed and approved by the Institutional Animal Care and Use Committee, University at Buffalo, The State University of New York. Twenty-four adult female rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing approximately 220 g were maintained on food pellets and water *ad libitum* and housed in an air-conditioned, humidity-controlled facility. A pure powder form of nifedipine (Sigma Chemical, St. Louis, MO) and sodium chloride (Fischer Scientific, Fair Lawn, NJ) were combined for the preparation of the nifedipine slurry. The injections were performed using a 10 ml syringe with 30 gauge needles, delivering a volume of 50 μl at each injection.

Twenty-four rats were assigned to three groups of eight rats each. Rats in Group 1 were given only saline (control group), rats in Group 2 received nifedipine (10 μg/ml) and rats in Group 3 received nifedipine (500 μg/ml). Those doses were chosen because they were comparable to gingival crevicular fluid concentrations observed in humans taking therapeutic doses of nifedipine (Ellis, *et al.*, 1993). Each animal received three similar injections one week apart. The injections were located at the maxillary and mandibular right side of the gingival papilla, distal to the upper and lower incisors. The animals were euthanized one week after the third and last injection via an intraperitoneal dose of phenobarbital (150-200 mg/kg). Tissue samples from the injection sites were obtained for routine

Mean number of cells per field positive for ED2 primary antibody

	Mean number of ED2-postive cells per field
Group 1	4.67 ± 1.68
Group 2	19.72 ± 13.28
Group 3	74.56 ± 28.23

hematoxylin & eosin staining and for immuno-cytochemistry.

Specimens for routine histology were fixed in 10% formalin at room temperature and slides 10 μm in thickness were obtained. Specimens for immunocytochemistry were frozen in liquid nitrogen and stored at -70°C. Four-micron cryostat sections were obtained from each frozen sample. For immunostaining, the frozen slides were brought to room temperature for 30 minutes and then fixed in acetone for 10 minutes. After fixation, the sections were dried for 3 minutes, then washed for 5 minutes in Tris-buffered saline (TBS, 20 mM Tris-HCL, 500 mM NaCl, pH 7.5), followed by incubation for 20 minutes in normal rabbit serum diluted 1:5 in TBS for blocking of non-specific background. The normal serum was successively removed by tapping, and the sections were treated with the primary monoclonal antibody (ED2) diluted 1:500 in TBS, as described by Kataoka *et al.* (2001) and as modified by us (Cohen *et al.*, 1991). This monoclonal antibody is specific for tissue-resident macrophages. All sections were washed in TBS for 5 minutes and then exposed for 30 minutes to biotinylated rabbit anti-mouse immunoglobulins (Dako Corp. Carpintera, CA) diluted 1:400 in TBS. After washing in TBS for 5 minutes slides were treated with avidin-biotin complex-alkaline phosphatase (Dako Corp. Carpintera, CA) for 30 minutes, then rinsed with TBS for 5 minutes. Localization of antigens was achieved by color development with a solution prepared from 2 mg naphthol AS-MX phosphate free acid (Sigma Chemical), 0.2ml N, N-dimethyl formamide (Sigma Chemical) 9.8 ml of 0.1 M Tris buffer pH 8.2 and 10 mg of Fast-Red TR salt (Sigma Chemical). The sections were analyzed by light microscopy after coverslipping with a water-based mounting medium (Dako Corp. Carpintera, CA).

Quantification of phagocyte subsets was performed in accordance with the methodology described by Honda *et al.* (1990) and as modified by us (Cohen *et al.*, 1991). Ten randomly selected fields on each specimen were analyzed by counting the number of cells labelled with the monoclonal antibody, at 200x magnification, from at least two duplicate connective tissue sections (i.e., two adjacent sections from the same specimen). The average number of cells per field positive for each antibody was obtained for each specimen. The values were computed and differences between the control group and the two treatment groups were determined by one-way analysis of variance (ANOVA) corrected for multiple comparisons.

Results

Macroscopic findings

Macroscopic examination did not reveal any alteration in the gross appearance of the rats' gingiva at the injection sites. The gingival tissue maintained its normal appearance with characteristic consistency, size and color, and without any sign of tissue enlargement. This observation was consistent irrespective of the group and the observational time.

Group 1: control Microscopic examination of the eight mandibular and eight maxillary specimens was consistent with normal microscopic anatomy. The rat incisor is characterized by continuous enamel formation that occurs on the buccal aspect of the tooth with the lingual aspect remaining exposed dentin (Graner *et al.*, 1995). No inflammatory infiltrate was detected in any of the control sections obtained from the mandibular arch.

Figure 1 is representative of the soft tissue attachment to dentin. The gingival sulcus is adjacent to the dentinal surface and the sulcular epithelium is keratinized with the epithelial layer ranging between 15 and 30 cells in thickness. Orthokeratinization was observed in each control section involving both oral and sulcular epithelium, and is consistent with the normal anatomy of the dento-gingival unit in rats (Graner *et al.*, 1995). The interface between the root and the periodontal soft tissues was characterized by a thin

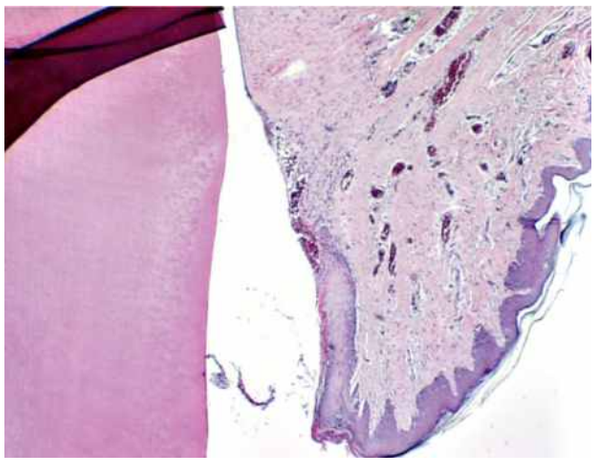

Figure 1. Photomicrograph of a gingival papillae from the maxillary arch of a rat from Group 1 (control). The gingival sulcus is adjacent to the dentinal surface and the sulcular epithelium is keratinized. (20X, H&E).

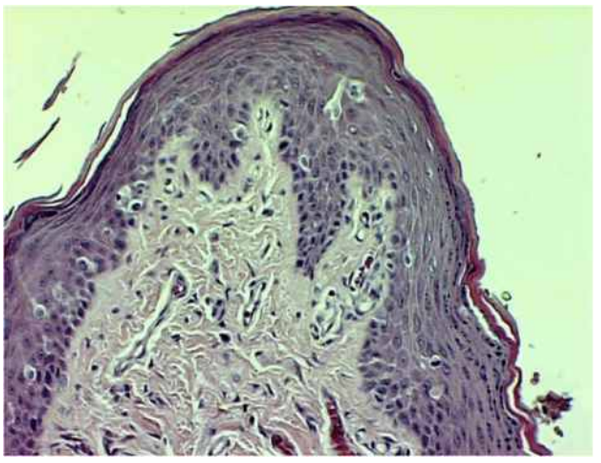

Figure 2. Photomicrograph of the buccal papilla from the mandibular incisor of a rat receiving high-dose nifedipine (Group 3). Sections from this group generally displayed an epithelial layer that maintained its integrity and was comparable to control sections. This section shows low cellularity and scattered lymphocytes. (100X, H&E).

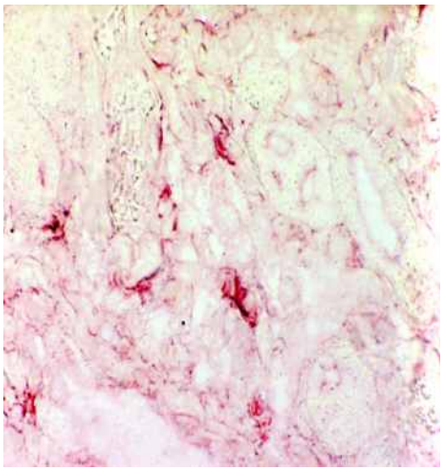

Figure 3. Photomicrograph of gingival connective tissue obtained from a rat receiving high-dose nifedipine (Group 3). The section was processed for immunocytochemistry using ED2-specific monoclonal antibodies with an avidin-biotin-alkaline phosphatase technique, and color developed with naphthol AS-MX phosphate/Fast-Red TR. (200X).

epithelial layer, 2 to 4 cells thick, representing the junctional epithelium. The connective tissue layer, or lamina propria, was without pathology in terms of collagen content, size and cellularity.

Group 2: low dose nifedipine. Specimens from Group 2 displayed a sulcus lined by orthokeratinized epithelium, 10-15 cell layers thick. The connective tissue exhibited normal extracellular matrix accumulation. Six out of eight specimens showed mild inflammatory infiltration, consisting of scattered polymorphonuclear neutrophils and a few lymphocytes.

Group 3: high dose nifedipine. Sections from this group displayed an epithelial layer that maintained its integrity and was comparable to control specimens. Its thickness ranged between 15 and 20 cell layers. The connective tissue was comparable to control sections in terms of collagen content (*Figure 2*). Mild inflammatory infiltration characterized by dispersed granulocytes and lymphocytes were detected in five of eight specimens. No frank alteration of papilla size was observed at the injection sites of any test specimens.

Immunocytochemical findings

Cells recognized by ED2 monoclonal antibody had a dendritic appearance, with small nuclei, little cytoplasm and multiple elongated processes as described in

previous studies (Cohen *et al.*, 1991; Kataoka *et al.*, 2001). The mean values of ED2-positive cells from the three groups are summarized in *Table 1*. ED2-positive macrophages were observed in specimens from all three groups, but the mean count of ED2-positive cells was significantly higher in Group 3 specimens (*Figure 3*), compared to the other two groups ($p < 0.0005$) and Group 2 compared to Group I ($p < 0.0005$). Differences between maxillary and mandibular samples were not evident, so those specimens were pooled for immunocytochemistry. A dose-dependent mean number of ED2-positive cells were noted in specimens from animals treated with nifedipine. The average number of ED2-positive cells was 4.6, 19.7 and 74.5 cells/field in Group 1, Group 2, and Group 3, respectively.

Discussion

Unfavorable gingival architecture may adversely affect plaque control and be unesthetic. Prosthetic rehabilitation of edentulous areas and interproximal spaces may be required to eliminate elongation of the clinical crown and/or appearance of black spaces (black triangles) between teeth caused by the loss of interdental papilla, which may be of concern for both patients and dental practitioners. Loss of interdental papilla may also have biological consequences, such as food impaction, increased dentinal sensitivity, and increased caries incidence.

At this time, soft tissue regeneration only can be achieved through surgical procedures such as gingival or connective tissue grafting to increase the volume and extent of soft tissue (Carnio, 2004). Results may be technique-sensitive and variable; patients may decline treatment due to cost, time or procedural factors (Ellis *et al.*, 1995). Although a less invasive and more cost-effective approach might result in greater patient acceptance, such procedures currently are not available. This study was performed to measure local gingival enlargement following repeated administration of nifedipine in the interdental papilla. Although significant changes in ED2-positive macrophages were noted immunocytochemically, gross alterations in gingival morphology were not observed.

Macrophages may play a role in the initial stages of fibroblast activation. Bellon *et al.* (2011) have found that macrophages may participate in human peritoneal fibrosis through the stimulation of fibroblast cell growth and cytokine production. Other studies have demonstrated the expression of fibroblast growth factors and receptors on macrophages and mast cells with an increase in collagen production (Akimoto *et al.*, 1999; Barron and Wynn, 2011; Prasse *et al.*, 2006; Takei *et al.*, 1989). Consequently, it is possible that macrophages may participate in intracellular communication during the first stages of nifedipine-induced gingival fibrosis. Although a decrease in gingival enlargement might be associated with discontinuing the use of nifedipine, further studies examining additional macrophage and fibroblast subsets would be indicated to more fully elucidate the precise mechanisms of inflammatory cell activation and to assess the permanence of drug-induced enlargement in this model.

To the best of our knowledge this study is the first attempt to induce drug-related gingival enlargement through local drug delivery. The presence of nifedipine in the gingival tissue may be required for production of gingival enlargement (Ellis *et al.*, 1992; Ellis *et al.*, 1995). However, it is possible that local administration under the conditions used in this study did not produce an effective concentration of nifedipine at the level of the dentogingival unit (Ellis *et al.*, 1995; Thomason *et al.*, 1998). The concentration of nifedipine utilized for each injection was derived from the concentration typically found in the gingiva of responder patients (i.e., patients taking nifedipine and developing gingival enlargement), but this may be more effective in humans compared to rats.

Two different concentrations were used (10 and 500 μg/ml) to analyze dose response. It is possible that a greater macroscopic effect might have been achieved by using higher nifedipine concentrations and increasing the frequency of drug delivery (daily or continuously instead of weekly), and/or by extending the length of the study.

We were able to demonstrate an inflammatory response through immunocytochemical analysis. Specimens from Group 3, the group injected with the highest nifedipine concentration, displayed a significantly greater number of ED2-positive cells compared to the other two groups. Specimens from Group 2 showed a significantly higher mean count of positive cells compared to Group 1. Collectively, our data suggest that repeated local injections of nifedipine with concentrations of 10 μg/ml and 500 μg/ml elicit an inflammatory response in the gingival connective tissue. Fibrosis, with collagen deposition, may occur as a result of prolonged inflammation (Ishida *et al.*, 1995). The duration of this study was only three weeks; a longer experimental period could lead to a more pronounced fibrosis, provided that inflammation persists for an extended period. More subtle changes in mononuclear cell subsets also might have been detected using a panel of monoclonal antibody probes specific for other macrophage subsets, as well as for T- and B-lymphocytes. Future studies will be directed towards further exploration of those variables.

Acknowledgment

There were no significant forms of support associated with this research. This study was supported in part by the Department of Periodontics and Endodontics, University at Buffalo, The State University of New York, School of Dental Medicine. There is no financial

relationship between any author and a commercial firm that would pose a conflict of interest.

References

Akimoto S, Ishikawa O, Iijima C, *et al.* Expression of basic fibroblast growth factor and its receptor by fibroblast, macrophages and mast cells in hypertrophic scar. *European Journal of Dermatology* 1999; **9**:357-362.

Barron L, Wynn TA. Interactions between fibroblasts, macrophages, Th2 and Th17 immunity regulate fibrosis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* [Epub ahead of print] Feb 3, 2011.

Bellon T, Martinez BT, Lucendo B, *et al.* Alternative activation of macrophages in human peritoneum: implications for peritoneal fibrosis. *Nephrology Dialysis Transplantation* [Epub ahead of print] Feb 15, 2011.

Birkedal-Hansen H. Role of cytokines and inflammatory mediators in tissue destruction. *Journal of Periodontal Research* 1993; **28**:500-510.

Bulut S, Uslu H, Ozdemir BH, *et al.* Analysis of proliferative activity in oral gingival epithelium in immunosuppressive medication induced gingival overgrowth. *Head & Face Medicine* 2006; **2**:13.

Carnio J. Surgical reconstruction of the interdental papilla using an interposed subepithelial connective tissue graft: a case report. *The International Journal of Periodontics & Restorative Dentistry* 2004; **24**:31-37.

Cohen RE, Aguirre A, Neiders ME, *et al.* Immunochemistry of low molecular weight human salivary mucin. *Archives of Oral Biology* 1991; **36**:347-356.

Ellis JS, Seymour RA, Monkman SC, *et al.* Gingival sequestration of nifedipine-induced gingival overgrowth. *Lancet* 1992; **339**:1382-1383.

Ellis JS, Seymour RA, Monkman SC, Idle JR. Disposition of nifedipine in plasma and gingival crevicular fluid in relation to drug-induced gingival overgrowth. *Journal of Periodontal Research* 1993; **28**:373-378.

Ellis JS, Seymour RA, Thomason JM, *et al.* Periodontal variables affecting nifedipine sequestration in gingival crevicular fluid. *Journal of Periodontal Research* 1995; **30**:272-276.

Fujii A, Kobayashi S. Nifedipine inhibits calcium uptake of nifedipine sensitive gingival fibroblasts. *Journal of Dental Research* 1990; **69**:100-386.

Gelfand EW, Cheung RK, Grinstein S, *et al.* Characterization of the role for calcium influx in mitogen-induced triggering of human T cells. Identification of calcium-dependent and calcium-independent signals. *European Journal of Immunology* 1986; **16**:907-912.

Graner E, Line SR, Jorge J, *et al.* Laminin and collagen type IV distribution and ultrastructure of the basement membrane of the gingiva of the rat incisor. *Journal of Dental Research* 1995; **30**:349-354.

Güncü GN, Cağlayan F, Dincel A, *et al.* Clinical and pharmacological variables as a risk factor for nifedipine-induced gingival overgrowth. *Australian Dental Journal* 2007; **52**:295-299.

Hardie DG. *Intracellular events. Biochemical messengers.* London: Chapman and Hall 1990; 191-247.

Honda H, Kimura H, Rostami A. Demonstration and phenotypic characterization of resident macrophages in rat skeletal muscle. *Immunology* 1990; **70**:272-277.

Ishida H, Kondoh T, Kataoka, M, *et al.* Factors influencing nifedipine-induced gingival overgrowth in rats. *Journal of Periodontology* 1995; **66**:345-350.

Kataoka M, Shimizu Y, Kunikyo K, *et al.* Nifedipine induces gingival overgrowth in rats through a reduction in collagen phagocytosis by gingival fibroblasts. *Journal of Periodontology* 2001; **72**:1078-1083.

Lin K, Guilhoto L, Yacubian E. Drug-induced gingival enlargement – Part 2. Antiepileptic drugs: not only phenytoin is involved. *Journal of Epilepsy and Clinical Neurophysiology* 2007; **13**:83-8.

Messing RO, Carpenter CL, Greenberg DA. Mechanisms of calcium channel inhibition by phenytoin: comparison with calcium channel antagonists. *Journal of Pharmacology and Experimental Therapeutics* 1985; **235**:407-411.

Nishikawa S, Nagata T, Morisaki I, *et al.* Pathogenesis of drug-induced gingival overgrowth. A review of studies in the rat model. *Journal of Periodontology* 1996; **67**:463-471.

Nishikawa S, Tada H, Hamasaki A, *et al.* Nifedipine-induced gingival hyperplasia: a clinical and *in-vitro* study. *Journal of Periodontology*1991; **62**:30-35.

Prasse A, Pechkovsky DV, Toews GB, *et al.* A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis via CCL18. *American Journal of Respiratory and Critical Care Medicine* 2006; **173**:781-792.

Seibel W, Yahia NA, McCleary LB, *et al.* Cyclosporine-induced gingival overgrowth in beagle dogs. *Journal of Oral Pathology & Medicine* 1989; **18**:240-245.

Shaker SA, Ayuob NN, Hajrah NH. Cell talk: a phenomenon observed in the keloid scar by immunohistochemical study. *Applied Immunohistochemistry & Molecular Morphology* 2011; **19**:153-159.

Shimizu Y, Kataoka M, Seto H, Kido J, Nagata T. Nifedipine induces gingival epithelial hyperplasia in rats through inhibition of apoptosis. *Journal of Periodontology* 2002; **73**:861-867.

Takei H, Yamada H, Hau T. Maxillary anterior esthetics. Preservation of the interdental papilla. *Dental Clinics of North America* 1989; **33**:263-273.

Thomason JM, Sloan P, Seymour RA. Immunolocalization of collagenase (MMP-1) and stromelysin (MMP-3) in the gingival tissues of organ transplant patients medicated with cyclosporin. *Journal of Clinical Periodontology* 1998; **25**:554-560.

Walters JD. Transmembrane signalling in periodontal mesenchymal cells: the linkage between stimulus and response. *Periodontology 2000* 1993; **3**:76-98.