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Original

Long-term effects of nifedipine on human gingival epithelium: a histopathological and immunohistochemical study

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Abstract: The chronic usage of nifedipine is associated with the appearance of gingival overgrowth (GO). The frequency of GO associated with chronic nifedipine therapy remains controversial and the possible subclinical effects of this drug on the gingival epithelium should be investigated. We investigated the epithelial proliferation index and apoptosis rate, and their association with epithelial enlargement. Proliferation (Ki67 and Cyclin B1) and apoptosis (BCL2, Bax and p53) markers were identified by immunohistochemistry in twenty-one samples of gingival tissue from patients undergoing chronic treatment with nifedipine and in eleven samples of gingival tissue from healthy patients who did not use drugs associated with GO (control). Our results show that the epithelial tissue of nifedipine users has considerably longer rete pegs compared to control (P = 0.01). However, the density of Ki67$^+$ and Cyclin B1$^+$ cells was similar in both groups. Regarding apoptosis, we found more BCL2$^+$ cells in the nifedipine group when compared to controls (P = 0.12). An increase in Bax$^+$ cells in the nifedipine group compared to control (P = 0.003) was also seen, and slightly lower levels of p53$^+$ expression were observed (P = 0.51). Our results suggest that the chronic use of nifedipine is not associated with subclinical changes in gingival tissue. (J Oral Sci 52, 55-62, 2010)

Keywords: cell proliferation; apoptosis; nifedipine; adverse effect; gingival overgrowth.

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Introduction

Nifedipine is a calcium channel blocker that is widely used in the treatment of cardiovascular disorders such as hypertension, angina pectoris and ventricular arrhythmias (1). It is known that chronic usage of this dihydropyridine is associated with the appearance of gingival overgrowth (GO) (2,3). Although it is not clearly understood exactly how GO develops, studies have shown unequivocal participation of fibroblasts and extracellular matrix in the pathogenesis of this unwanted effect (4-6).

An increase in epithelial thickness with elongated rete pegs was described following the first reports of nifedipine-induced gingival overgrowth (NIGO) (7,8). More recently, studies have shown that the epithelial thickness encountered in GO could be associated with an increase in the mitotic activity of the epithelium, as measured by the expression of Ki67 and Cyclin B1 proliferation markers (9,10). Ki67 is a non-histonic nuclear protein that is present throughout all the active phases of the cell cycle (G1, S, G2, and M), but is absent from resting cells (G0), and reaches its peak

concentration in phases G2 and M (11). Cyclin B1 is a cytoplasmic protein that, when combined with another protein known as Cdk 1, forms the MPF (M-phase Promoting Factor). Cyclin B1 begins to accumulate during S and G2, reaching a peak concentration in mitosis, particularly in metaphase (12). The Ki67 and Cyclin B1 markers are considered to be good indicators of the proliferative activity of a cell population.

Recent studies have shown that nifedipine is able to block the apoptosis of endothelial cells (13,14) and keratinocytes (15-17). Several studies have been carried out in order to investigate apoptosis in NIGO (13-18). These studies have investigated BCL2, Bax and p53. The BCL2 family can be classified into two functionally distinct groups: anti-apoptotic proteins and pro-apoptotic proteins (18). BCL2, an anti-apoptotic protein, is known to regulate the apoptotic pathways and protect against cell death, while Bax is a pro-apoptotic protein of the same family that is expressed abundantly and selectively during apoptosis and promotes cell death (18). Another major regulator of apoptosis induction is the tumor suppressor gene, p53, which acts via the activation of apoptosis-inducing genes such as Bax (18).

The prevalence of GO among nifedipine users varies greatly (19,20). Estimates of the prevalence of nifedipine-induced gingival overgrowth range from 6.3 to 83%. This broad range could be attributed to the methods used to assess GO or the population sampling technique (21). Given that the frequency of GO associated with chronic nifedipine therapy is controversial, the possible subclinical effects of this drug on the gingival epithelium should be investigated. The aim of this study is therefore to investigate the possible microscopic changes in the gingival epithelium of nifedipine users and to correlate these findings with the mitotic activity and apoptosis rate of keratinocytes. To measure the epithelial proliferation index and apoptosis rate of keratinocytes, cellular identification of the Ki67, Cyclin B1, BCL2, Bax and p53 proteins was carried out using an immunohistochemical technique.

Materials and Methods

Twenty-one patients who were treated with nifedipine for hypertension and who had received no other GO-inducing drugs (hydantoin-derived drugs, cyclosporin or other calcium channel blockers) attended at the Goiás Oral Disease Center of the Federal University of Goiás. The GO index was evaluated through clinical examination according to Inglés et al., and was divided into four categories. Categories (0) and (1) show normal gingival dimensions, while categories (3) and (4) indicate clinically evident GO (22). Clinical features and demographic data are presented in Table 1. Patients underwent biopsies taken from a zone of keratinized mucosa 3-4 mm apical to the gingival margin during a tooth extraction procedure. Control samples were collected from eleven healthy patients who were not taking medication and were undergoing a crown lengthening procedure. Efforts were made to perform biopsies at clinically healthy gingival sites. Each individual gave written informed consent for the use of their specimens and the protocol was approved by the Ethics Committee of the Federal University of Goiás. The specimens were fixed in 10% formalin and standard dehydration and paraffin-wax embedding procedures were used. Sections (5 μm) were cut in a microtome and adhered to glass slides. Hematoxylin and eosin (HE)-stained slides were prepared using standard methods and were evaluated by light microscopy. Using an integration graticule (CARL ZEISS-4740680000000-Netzmikrometer, ×12.5 Carl Zeiss, Göttingen, Germany), all existing rete pegs in each specimen were measured.

Immunohistochemistry

In order to assess the growth fractions of the 32 cases, an immunohistochemical study on the expression of Ki67 (clone MM1, Novocastra, Newcastle Upon Tyne, UK) diluted at 1:100, Cyclin B1 (clone 7A9, Novocastra, Newcastle, UK) at 1:40, BCL2 (clone 124, DAKO, Glostrup, Denmark) at 1:500, Bax (A3533, DAKO, Glostrup, Denmark) at 1:500, and p53 (clone DO-7, Novocastra, Newcastle Upon Tyne, UK) at 1:200 was carried out. Briefly, paraffin-embedded tissues were sectioned (3 μm), and serial sections were collected on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma Chemicals, St. Louis, MO, USA). Sections were deparaffinized by immersion in xylene, and this was followed by immersion in alcohol and incubation with citrate buffer (pH 6.0; SIGMA, P4809, St. Louis, MO, USA) or EDTA buffer for the expression of BCL2 (pH 9.0, Tris Base) for 40 min at 95°C for antigen retrieval. Next, sections were immersed in 3% hydrogen peroxide diluted in Tris buffered saline (TBS) (pH 7.4) for 20 min. Soon afterward, sections were blocked by incubation with 3% normal goat serum diluted in distilled water, at room temperature, for 20 min. Slides were then incubated overnight with primary antibodies at 4°C in a humidified chamber.

After washing in TBS, sections were treated with labeled streptavidin-biotin (LSAB) kits (K0492, DAKO, Denmark). Sections were then incubated in 3,3'-diaminobenzidine (DAB) in a chromogen solution (K3468, DAKO, USA) for 2 to 5 min at room temperature. Finally, sections were stained with Mayer's hematoxylin and were covered.

Table 1 Demographic and clinical characteristics of patients (means, standard deviations, minimum and maximum values)

	Nifedipine group (n = 21)	Control group (n = 11)
Age (years)	57.2 ± 13.1 (29-74)	41.5 ± 9.7 (24-51)
Gender (men/women)	8:13	6:5
Dose of nifedipine (mg/day)	33.5 ± 10.3 (10-40)	-
Duration of medication (months)	52.3 ± 36.7 (10-144)	-

Negative controls were obtained by the omission of primary antibodies, which were substituted with 1% PBS-BSA or non-immune mouse (X0910, DAKO, USA) serum.

Quantitative analysis

The number of Ki67$^+$, Cyclin B1$^+$, BCL2$^+$, Bax$^+$ and p53$^+$ cells was determined separately, in the basal and suprabasal layer of epithelial tissue of both the nifedipine and control groups, using an integration graticule (CARL ZEISS-4740680000000-Netzmikrometer, ×12.5 Carl Zeiss, Göttingen, Germany). All cells were counted in ten representative and consecutive microscopic high-power fields (×400) and at this magnification each field (integration graticule) had an area of 0.0961 mm^2. Ten non-contiguous and non-overlapping squares were randomly selected for cell counting in the basal and suprabasal layers of the epithelium. The mean of stained cells was obtained by dividing the number of positive cells by the total area (0.961 mm^2). All measurements were made by the same examiner following calibration.

Statistical analysis

Results are expressed as the mean of positive cells ±standard deviation (SD) of n observations per mm^2. Data were analyzed using the Mann-Whitney's *U*-test in order to compare the two groups. All statistical tests were performed using SPSS 10.0 software (SPSS 10.0.1 for Windows, SPSS Inc., Chicago, IL, USA). Significance was set at 0.05.

Results

The patients in this study (eight males and thirteen females with a mean age of 57.2, range: 29-74 years) received treatment with nifedipine (mean length of treatment of 52.3 months; range of 10-144 months) at a mean dose of 33.5 mg/day (range 10-40 mg/day) (Table 1). The GO index revealed that all patients taking nifedipine (n = 21) presented with normal gingival dimensions, categories (0) and (1), according to the index described by Inglés et al. (22).

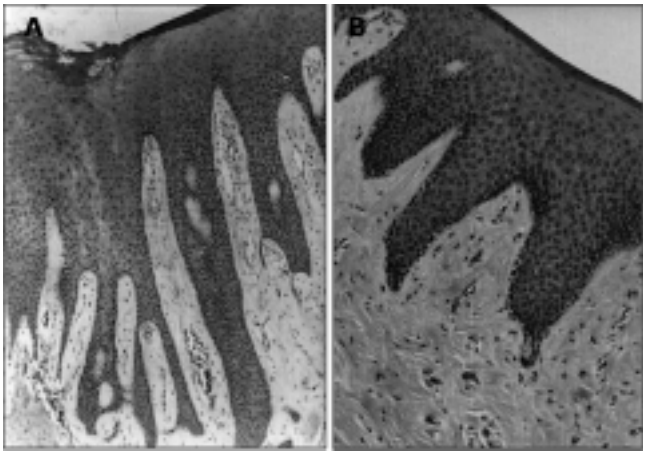

Fig. 1 Photomicrographs showing gingival tissues with elongated rete pegs in nifedipine users (A) and with short rete pegs in control patients (B). In both groups, the connective tissue contains numerous fibroblasts and collagen fibers, and lacks inflammatory infiltrates (Hematoxylin and Eosin, original magnification ×250).

The microscopic features observed in the treated and control groups were similar. The gingival samples showed a stratified squamous epithelium that was predominantly parakeratinized with areas of acanthosis, and large variations in the size of rete pegs. The underlying connective tissue exhibited a variable number of fibroblasts, typical collagen bundles and blood vessels. Inflammatory infiltrates were very scarce or non-existent in both groups. The length of the rete pegs was calculated as the distance between the granular and basal layers of epithelial tissue (based on Bulut et al., 2006) (9). Thus, the rete pegs were placed in two categories: short rete pegs (length ≤ 0.75 mm) and long rete pegs (length ≥ 0.75 mm). The percentage of long rete pegs in the two groups differed considerably. The samples from the nifedipine users contained a significantly higher percentage of long rete pegs (P = 0.01, Mann-Whitney's *U*-test) than those from the control group (18.9% in the nifedipine group vs. 12.0% in the control group) (Fig. 1).

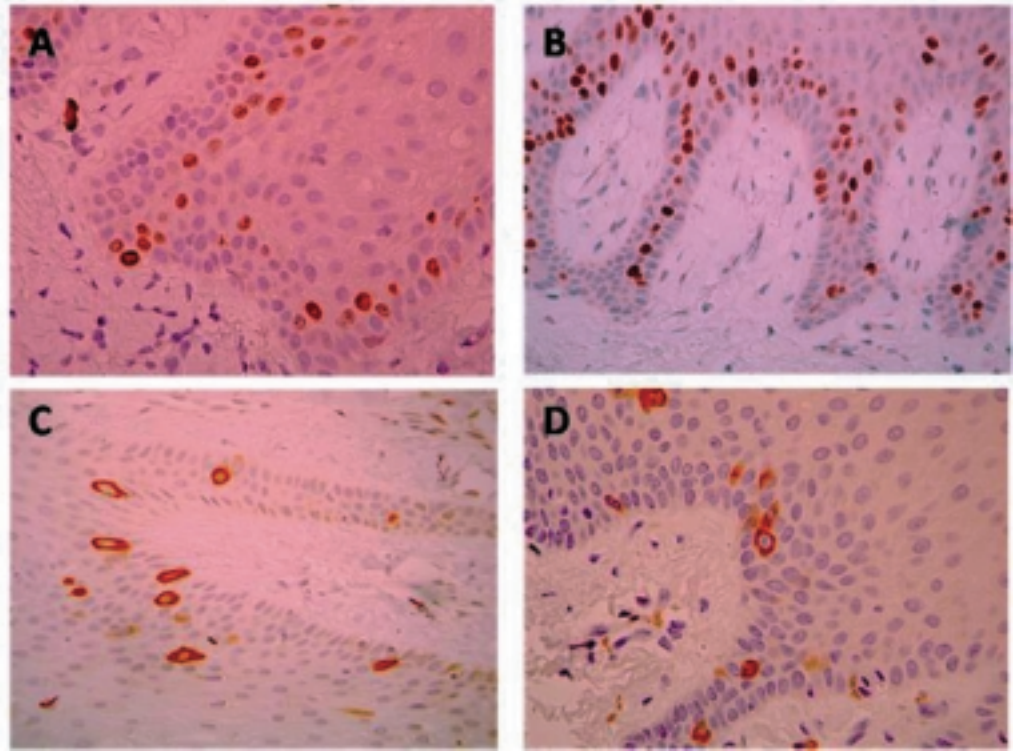

Fig. 2 Immunohistochemical staining for Ki67 (A and B) and Cyclin B1 (C and D) in basal and suprabasal keratinocytes of nifedipine users (A and C) and control patients (B and D) (Immunohistochemical staining, original magnification ×400).

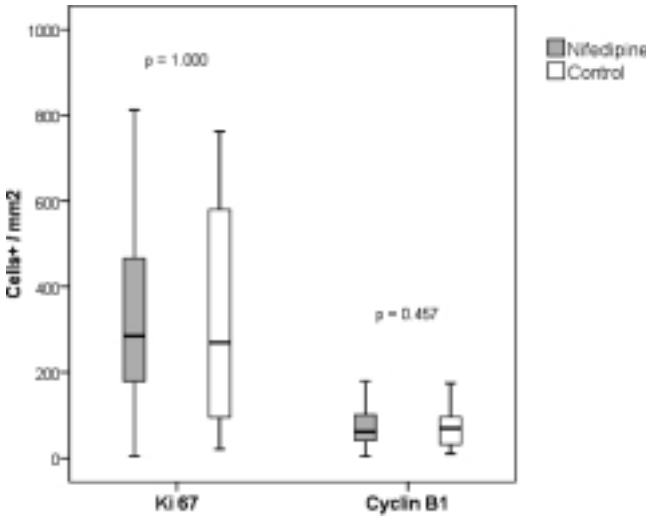

Fig. 3 Densities of Ki67$^+$ and Cyclin B1$^+$ cells in the nifedipine (n = 21) and control (n = 11) groups. Results are expressed as mean cells/mm^2 ± SD.

Ki67$^+$ cells with characteristic nuclei were immunohistochemical detected in the basal and suprabasal layers of both the nifedipine group and the control group (Figs. 2A and B). Quantitative analysis revealed no significant differences between the number of Ki67$^+$ cells in the two groups (330.6 ± 232.6 in the nifedipine group and 333.4 ± 277.5 in the control group, Mann-Whitney's U-test, P = 1.0) (Fig. 3). With respect to the expression of Cyclin B1, a small number of positive cells with characteristic cytoplasmatic staining was observed (Figs. 2C and D). As found on Ki67 analysis, immunostaining of Cyclin B1 protein was similar in both groups (76.9 ± 49.6 in the nifedipine group and 64.8 ± 47.5 in the control group; Mann-Whitney's U-test, P = 0.45) (Fig. 3).

With regard to the anti-apoptotic (BCL2) and pro-apoptotic proteins (Bax and p53), we found strong staining of BCL2$^+$ cells scattered in the basal and suprabasal layers of the nifedipine group, while control epithelia contained fewer BCL2$^+$ cells (Figs. 4A and B). The number of BCL2$^+$ cells was higher in the nifedipine group than in the control group, but no significant differences were seen between the two groups (104.4 ± 33.2 and 57.0 ± 49.9 respectively, Mann-Whitney's U-test, P = 0.12) (Fig. 5).

The expression of a pro-apoptotic protein (Bax) was observed in the basal and suprabasal layers of epithelial cells (Figs. 4C and D). The number of Bax$^+$ cells/mm^2 was

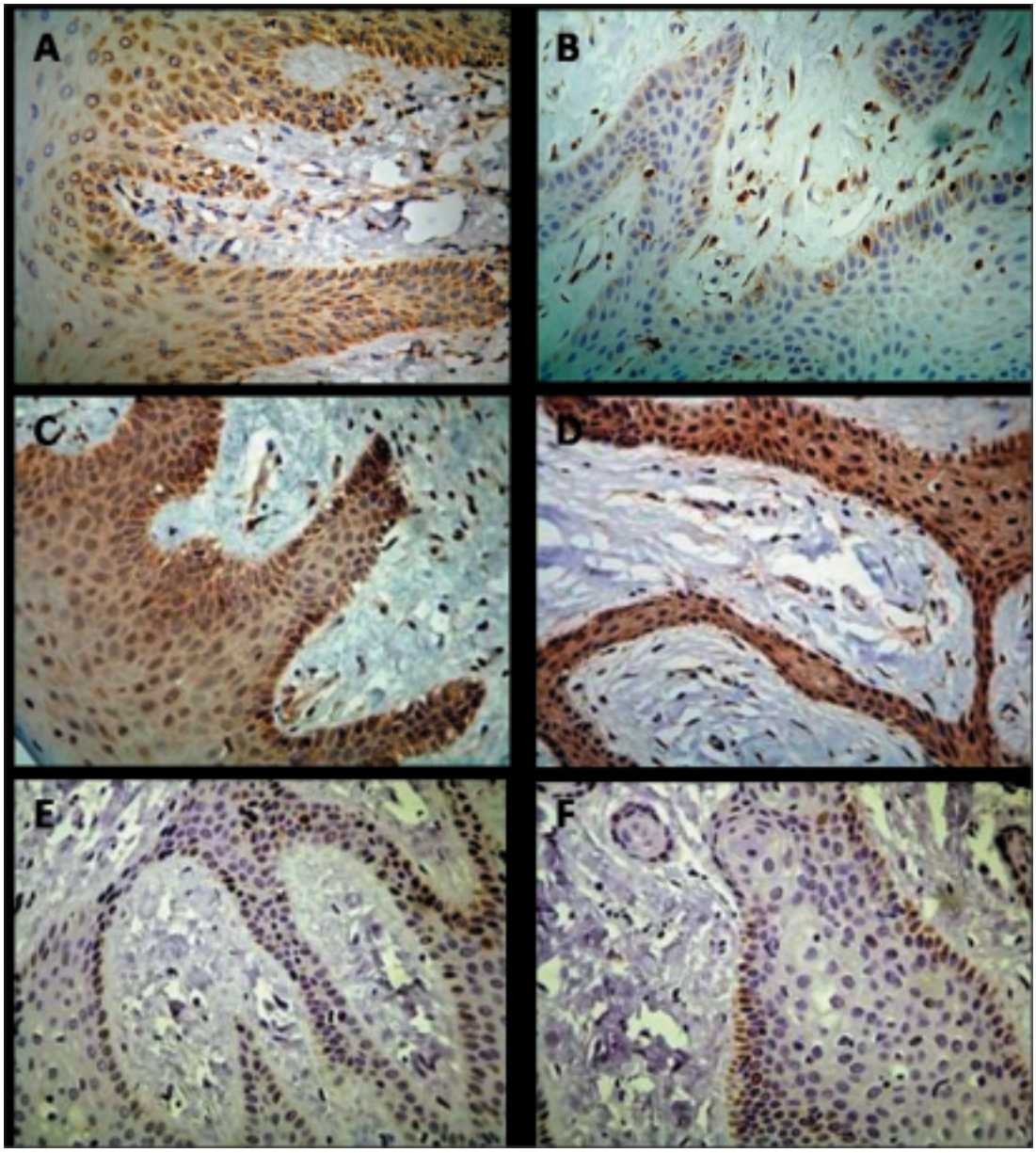

Fig. 4 Immunohistochemical staining for BCL2 (A, B) Bax (C, D) and p53 (E, F) in basal and suprabasal keratinocytes of nifedipine users (A, C and E) and control patients (B, D and F) (Immunohistochemical staining, original magnification ×100).

significantly higher in the nifedipine group when compared to the control group (143.1 ± 27.2 and 76.0 ± 54.5 respectively; Mann-Whitney's *U*-test, $P = 0.003$) (Fig. 5). Moreover, this study showed a sporadic distribution of p53 protein in the suprabasal layers of the nifedipine group (Figs. 4E and F). The mean number of $p53^+$ cells/mm^2 in the nifedipine and control groups was 3.2 ± 2.7 and 10.9 ± 16.7, respectively (Fig. 5).

Discussion

In the present study, microscopic analysis revealed marked epithelial changes in the gingival tissue of nifedipine users. Although we found no clinically significant GO, the gingival tissue of patients using nifedipine exhibited a higher percentage of elongated rete pegs when compared to the control group (rete pegs longer than 0.75 mm), indicating a subclinical manifestation in nifedipine users. Calculation of the length of the rete pegs was based on Bulut et al. (9), because there have been few studies related to this topic. Our data are consistent with those reported by Barak et al. in 1987 (7).

On the other hand, Ki67 and Cyclin B1 expression were

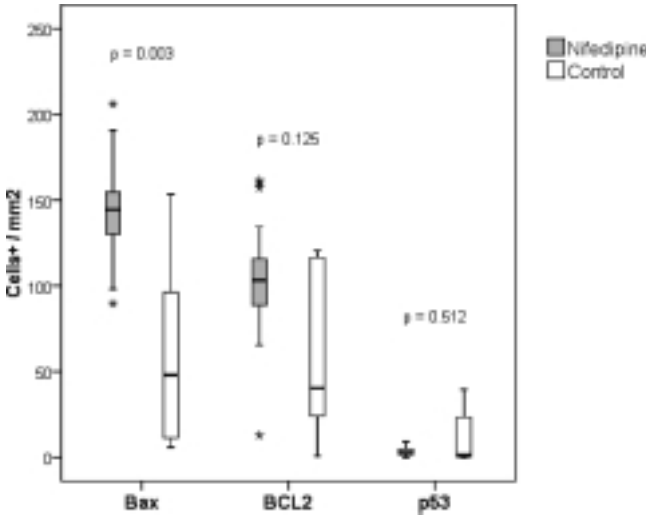

Fig. 5 Densities of BCL2$^+$ and Bax$^+$ and p53$^+$ cells in nifedipine (n = 21) and control (n = 6). Results are expressed as mean cells/mm^2 ± SD. Difference between nifedipine and control groups was significant for Bax$^+$ expression (P = 0.003).

similar in the gingival epithelia of nifedipine users and control patients. With reference to this data, our findings differ from those of other studies, which found that gingival samples extracted from patients taking nifedipine displayed significantly higher levels of Ki67 expression when compared to control patients (10,23). This difference may be explained by the fact that in our study, most samples did not show gingival enlargement, while in the other above-cited studies (10,23), the authors used samples obtained from hyperplastic gingival tissue (moderate to severe degrees of GO).

One can thus hypothesize that an increase in the mitotic activity of epithelial cells, as described by Saito et al. (23), Nurmenniemi et al. (10) and Mesa et al. (24), occurs only when moderate or severe GO is present. It can also be supposed that, as there is no clinically evident gingival enlargment, the gingiva of nifedipine-treated patients showed an epithelial proliferation rate comparable to that of healthy patients.

Several studies have established that the proliferative activity of keratinocytes can be mediated by mitogenic factors originating from connective tissues, such as keratinocyte growth factor and scatter factor (25-27). It can be postulated that the high proliferation rate of keratinocytes described previously did not occur due to direct action of the drug in the epithelium, but by interaction between the fibroblasts and epithelial cells. In responder patients, there may be some subpopulations of fibroblasts that react to the drug in a direct or indirect manner. This fibroblastic activity can explain various events in drug-induced gingival overgrowth, including the increased mitotic activity of keratinocytes (4-6,28-31).

It is interesting to note that the epithelial changes detected by our microscopic analysis were not associated with proliferative activity. In 1999, Saito et al. (23) observed a surprising decrease in Ki67 expression in the elongated rete pegs of GO patients. This finding reinforces the assumption that the enlargement of rete pegs is not positively related to the epithelial proliferation index. Recent studies have shown that nifedipine is able to block the apoptosis of endothelial cells (13,14). Similarly, in GO studies, Saito et al. (16), Shimizu et al. (17) and Handajani et al. (15) demonstrated that nifedipine has an inhibitory effect on the apoptosis of keratinocytes. Using these studies as a basis, we also decided to investigate the expression of apoptosis markers in the gingival epithelia of nifedipine users and control patients.

It is known that apoptosis is a complex phenomenon that involves a cascade of biochemical events. One of the key events in the apoptosis process is a rise in intracellular calcium concentration (32,33). It can be hypothesized that this kind of inhibitory effect may even happen directly in the epithelium. Nifedipine is able to block the calcium influx in keratinocytes and hinder the entry of these cells into apoptosis (17).

Although the mechanisms of this release of apoptosis inhibition are not clear, blockade by nifedipine of one or more steps in the cascade may result in a decrease in the apoptosis rate (14). According to Shimizu et al., gingival keratinocytes grown in culture under low levels of calcium express BCL2, which inhibits apoptosis; in contrast, keratinocytes grown under high levels of calcium express Bax, which induces apoptosis (17).

In the present study, we observed more BCL2$^+$ cells in the nifedipine group when compared to the control group. Similar results were seen in the studies of Handajani et al. and Saito et al., who found that the numbers of BCL2$^+$ cells in the gingiva of nifedipine-treated animals and patients, respectively, were higher than in control groups (15,16). Thus, it could be speculated that accumulation of nifedipine in the gingival tissues may specifically induce excess intracellular signals in the epithelial cells, leading to overexpression of BCL2 protein. In summary, the precise regulatory role of BCL2 in nifedipine-induced gingival hyperplasia remains unclear, as can be seen in this study.

We also found a significant increase in Bax$^+$ cells in the nifedipine group when compared to the control group. Our results suggest that apoptosis occurs in the nifedipine group in contrast to the control group, although it is known

that gingival epithelial overgrowth is common in nifedipine users (2,3,21). However, in this specific research, gingival samples were only selected from nifedipine users without clinical gingiva enlargement, as our intention was to observe the subclinical effects of nifedipine in epithelial tissue.

This positivity of Bax may be explained by the fact that the pro-apoptotic protein Bax binds to the anti-apoptotic protein BCL2, leading to the inactivation of the anti-apoptotic function of BCL2 and Bax plays a key role in mediating the apoptotic program in response to genotoxic stress, thereby preventing the development of gingival overgrowth (34). It has been proposed that a balanced ratio between BCL2/Bax regulates the apoptotic pathway through the dimerization of the two proteins, either with one another or with themselves (34). However, to the best of our knowledge, there have been few studies to date examining the relationship between BCL2 and Bax expression and gingival epithelial overgrowth.

In the literature, two possibilities have been proposed in order to explain the immunodetection of p53 protein expression. One is that wild-type p53 protein is overexpressed immediately after DNA damage (17). The other is that the half-life of mutant p53 protein is prolonged and it remains within the nuclei. In normal tissues, wild-type p53 is not detected under normal conditions, as a result of its short half-life. However, in response to genotoxic agents, wild-type p53 accumulates and induces apoptosis (17). In our study, we employed an anti-p53 antibody that recognizes both the mutant and wild-type p53 protein. However, both types of p53 accumulation suggest the genomic alteration of p53 or genes that encode negative regulators of p53.

In contrast to Haniastuti et al. (35), who observed the overexpression of p53 protein in the gingival epithelial cells of nifedipine-treated animals, we found a sporadic distribution of $p53^+$ cells in the suprabasal layers of the nifedipine group. The vast majority of patients ($n = 21$) taking nifedipine exhibited low levels of p53 expression; thus, p53 was not detected in normal tissues.

In conclusion, the enlargement of epithelial rete pegs in nifedipine users does not occur either due to mitotic activity or as a result of inhibition of apoptosis in keratinocytes. The results of this study suggest that chronic usage of nifedipine is not associated with the appearance of subclinical changes in gingival tissue. The factors that are involved in the origin of GO therefore remain unclear and require further investigation.

Acknowledgments

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